Constructing a synthetic microbial community based on *Serendipita indica* thiamine auxotrophy

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Contents

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysed) was carried out by the author except in the cases outlined below:

1) Maintenance and generation of fresh *S. indica* DSM 11827 culture were carried out in Dr. Patrick Schäfer's lab.
2) *B. subtilis* NCBI 3610 cryostock was obtained from Dr. Munehiro Asally's lab.
3) *A. thaliana* Col-0 seeds were obtained from Dr. Patrick Schäfer's lab.
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Abstract

Natural microbial communities act as metabolic conversion systems in soil, oceans, animal guts and other environments, provide essential nutrition for animals and plants, and drive global biogeochemical cycles. Such functions rely on complex interactions among microbes with different genotypes and metabolic capabilities. In order to achieve a deeper understanding of microbial communities and to further engineer synthetic communities, it is necessary to identify the metabolic interactions among key species, and characterise how these interactions are affected by different environmental factors. Deciphering the physiological basis of species-species and species-environment interactions in spatially organized microbial communities requires bottom-up approaches through assembling ecologically and functionally relevant species.

To this end, the work herein focuses on a defined system to study the metabolic interactions in a spatial context between the plant-beneficial endophytic fungus *Serendipita indica* and the soil-dwelling model bacterium *Bacillus subtilis*. Focusing on the growth dynamics of *S. indica* under defined conditions, it was discovered that this organism was auxotrophic to thiamine, a co-factor for essential reactions in the central carbon metabolism. Furthermore, it was found that the growth of *S. indica* was restored in thiamine-free media when co-cultured with *B. subtilis*. However, the success of this auxotrophic interaction was determined by the spatial and temporal organization of this two-species synthetic community; the beneficial impact from *B. subtilis* to *S. indica* was only possible when inoculation of *B. subtilis* was separated from that of *S. indica* either in time or space. The microscopy analyses were performed and a microfluidic system was developed to investigate the real-time community interaction and fungal growth at single cell level. The time-lapse imaging

data of interactions between *S. indica* and *B. subtilis* as well as *S. indica* spore germination were analysed, and obtained a fine characterisation of the growth dynamics of *S. indica*.

The following work described the thiamine auxotrophy of *S. indica*, the key auxotrophic interaction between *S. indica* and *B. subtilis* and the importance of spatial and temporal organization for the success of auxotrophic interactions. These discoveries contribute to the understanding of *S. indica* growth, allow the controlled investigations of fungal-bacterial interactions and have implications for the engineering of functional synthetic communities with plant beneficial microbes.

Abbreviations

A. thaliana	*Arabidopsis thaliana*
AMF	arbuscular mycorrhizal fungi
ATS	*Arabidopsis thaliana* salts (growth medium)
BAS	branching absorbing structures
BLAST	basic local alignment search tool
B. subtilis	*Bacillus subtilis*
CM	complete medium
Gln	glutamine
HPLC	high-performance liquid chromatography
IC	ion chromatography
NH_4^+	ammonium
PDMS	polydimethylsiloxane
PES	polyethersulfone
PTFE	polytetrafluoroethylene
S. indica	*Serendipita indica*
TCA cycle	tricarboxylic acid cycle

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Chapter 1 Introduction

1.1 Microbial community

The quote, "No man is an island, entire of itself; every man is a piece of the continent, a part of the main. (John Donne, Meditation XVII,1623)" suggests that alone a human cannot be self-sufficient; they must be part of a community. The same holds true for the microbial world. Microbes (for example bacteria, archaea, microalgae, fungi and protists) occupy every corner of the earth habitat in large numbers and therefore, they will inevitably interact directly or indirectly with each other and with other organisms. In addition, similarly to the concept of communities in plant or animal ecology, microbial communities are defined as the groups of microbes sharing a common living space (Connell, 1972; Konopka, 2009).

The members of microbial communities interact with each other in different ways, which can be categorised into six basic motifs as follows (Großkopf and Soyer, 2014) (Figure 1-1). Firstly, commensalism, in which one organism benefits from another one, and the latter experiences no benefit or harm. An example of this motif is found in situations like wastewater treatment, where one species feeds on the product of another species (Pérez *et al.*, 2015). The second motif, cooperation, that is two organisms mutually benefitting from each other and is commonly observed in the process of degrading complex organic matter during methane bioproduction (Schink, 1997). Thirdly, competition, in which two organisms try to acquire the same limited resource, resulting in one or both species impaired or eliminated (Gause, 1934; Hardin, 1960). For example, there are microbes reported to release antibiotics that can eliminate their competitors (Ghoul and Mitri, 2016). Fourthly, predation, in which one organism invades or consumes another as reported for *Bdellovibrio bacteriovorus*, which can attack and kill *Escherichia coli* (Martin, 2002). The fifth motif, amensalism, in which one organism causes harm to another without taking any benefits or cost is

observed, for example, when *Pseudomonas taetrolens* is inhibited by *Lactobacillus casei* in cheese production (García *et al.*, 2017). Finally, neutralism, is when two organisms do not significantly affect each other. Additionally, the neutralism-based interactions do not necessarily affect organisms involved (Lidicker, 1979).

Usually, combinations of these basic motifs of interactions are found within a microbial community. For example, both cooperation and predation are observed in the microbial community of the active sludge from wastewater treatment. Cooperation is observed between ammonia oxidisers and nitrite oxidisers, such as ammonia oxidisers producing nitrite that can be used by nitrite oxidisers, and in return, nitrite oxidisers consume nitrite and reduce the potential toxicity of nitrite accumulation in the system (Pérez *et al.*, 2015). Predation is observed when chemoautotrophy nitrifying bacteria (such as the ammonia oxidisers and nitrite oxidisers) are attacked by heterotrophs such as protozoa, rotifers and nematodes (Moussa *et al.*, 2005; Dolinšek *et al.*, 2013).

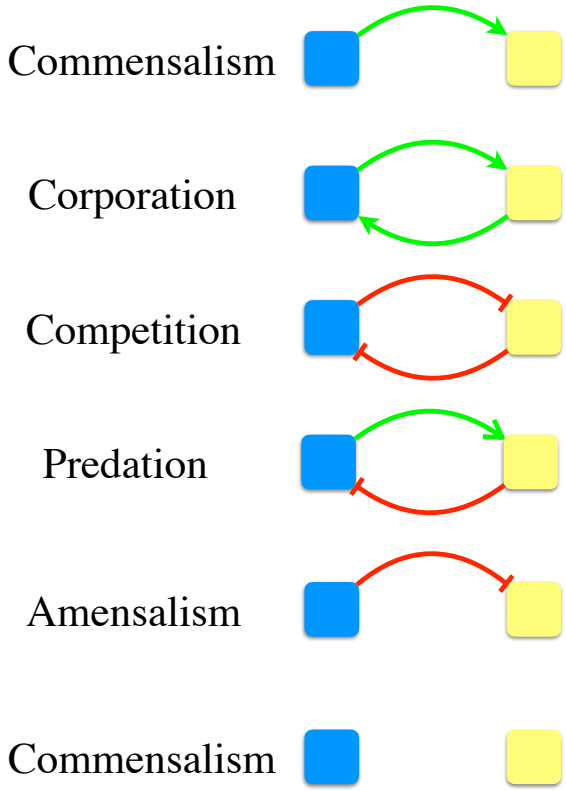


Figure 1-1: Basic motifs of microbial interactions. Blue and yellow squares represent two different microbial species. Green connection indicates interaction with directional benefit. Red connection indicates interaction with directional inhibition. The image is modified from (Großkopf and Soyer, 2014).

The way microbes interact with each other determines the overall structure and dynamics of a community (Little *et al.*, 2008; Großkopf and Soyer, 2014). Microbial communities are involved in many critical processes (Falkowski *et al.*, 2008), such as carbon and nitrogen recycling in oceans, establishing human gut or skin health, production of renewable energy and wastewater treatment. Analysing the composition and the interactions among the species in a microbial community could help gain the understanding of the overall structure and function of that community.

1.2 Rhizosphere and root microbiota

In 1904, Lorenz Hiltner defined the rhizosphere as a specific region in soil that surrounds the root system and is influenced by living roots (Hartmann *et al.*, 2008). Due to the variance of root shape and length, rhizospheres are not strictly defined by size, but instead by the concept on gradual changes in chemical, biological and physical properties (Abbott and Murphy, 2003). Plants release rhizodeposits (e.g. carbohydrates, amino acids) to the rhizosphere to attract and hence, benefit from microbes (Figure 1-2) (Hartmann *et al.*, 2008; McNear, 2013). The microbes in this root-soil interface are directly influenced by living plant roots. Those microbes living inside or on the surface of root together form the community known as the root microbiota (Bulgarelli *et al.*, 2013).

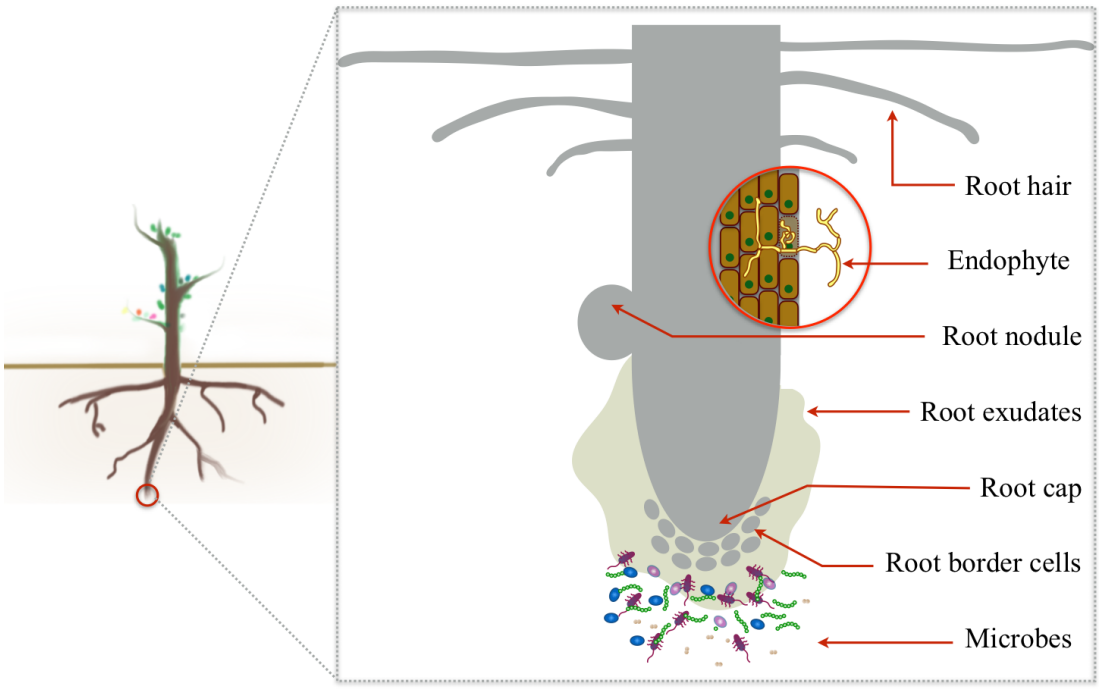


Figure 1-2: Schematic of rhizosphere. Grey dotted box indicates the rhizosphere region. Red circles indicate the magnification view of the original parts. Endophyte (microbe lives within plant) is indicated in yellow filaments. The image is modified from (Philippot *et al.*, 2013).

Microbes colonise the rhizosphere to obtain organic nutrients, which are generally very limited in bulk soil (Wardle, 1992). As a result, the microbial biomass and activity are much higher in the rhizosphere than in bulk soil (Minz *et al.*, 2013). The growth of various species leads to complex interactions among those species and with plants. In the rhizosphere, there are many microbes that will have neutral effects on plants, but there are also beneficial microbes and pathogens. Beneficial microbes can support plants and promote plant growth, while pathogens invade and kill plants or reduce plant growth (Raaijmakers *et al.*, 2009). There are extensive studies about these positive and negative effects (Babalola, 2010; Raaijmakers *et al.*, 2009; McNear, 2013), revealing the importance of root microbiota to plants.

Based on 16s rRNA gene sequencing, the compositions of different root microbiota are determined. For example, while *Proteobacteria* and *Firmicutes* are dominant among 1917 taxa detected in oat (DeAngelis *et al.*, 2009), potato roots from field soil are enriched in *Actinobacteria* and *Alphaproteobacteria* (İnceoğlu *et al.*, 2011), and *Proteobacteria* cover 36 - 40 % of the total sequencing reads in the root microbiota of *Rhizophora mangle* (red mangrove) (Gomes *et al.*, 2010). The composition of root microbiota can be influenced by both plants and edaphic factors (e.g. physicochemical soil characteristics) (Minz *et al.*, 2013; Bulgarelli *et al.*, 2013). Studies have reported many specific root microbiota compositions based on variables such as plant species (Long, 1989), growth stage (Van Overbeek and Van Elsas, 2008), health status (Yang *et al.*, 2001), soil type (Marschner *et al.*, 2001) and soil nutrient composition (Ofek *et al.*, 2009).

Root microbiota is important for plant growth (Raaijmakers *et al.*, 2009; Bulgarelli *et al.*, 2013), and its composition can be influenced by complex factors (Minz *et al.*, 2013; Bulgarelli *et al.*, 2013). Therefore, more detailed studies to

decipher these factors is a prerequisite for the ability to influence root microbiota towards improvements in plant growth.

1.3 Plant beneficial microbes

As discussed in section 1.2, root microbiota could have impacts on plant growth. However, it is less clear how these impacts arise. It has been suggested that there are key plant beneficial microbes within the rhizosphere that are the essential mediators of beneficial impacts on plants (Philippot *et al.*, 2013; Yadav *et al.*, 2015). For example, certain microbes (in particular the soil-dwelling bacteria and the endophytic fungi) can promote plant growth, help plants in assimilating nutrition, or in the protection against plant pathogens (Raaijmakers *et al.*, 2009; Mendes *et al.*, 2013).

There are different ways beneficial microbes associate with plants; plant beneficial bacteria can live freely in soil (Lucy *et al.*, 2004), associate with plant roots (such as covering the root surface to enlarge the contact area with the plant) (Babalola, 2010) (Figure 1-3 A), or establish intracellular structures (such as root nodules inside root cells) for nutrient exchange (Vessey, 2003) (Figure 1-3 B). Plant beneficial fungi can colonise host root tissue extracellularly (ectomycorrhizal fungi) (Figure 1-3 C) or intracellularly (endophytes such as endomycorrhizal fungi) (Figure 1-3 D) (Weiss *et al.*, 2016), in addition, there are also free living fungi (such as *Trichoderma spp.*) (Harman *et al.*, 2004).

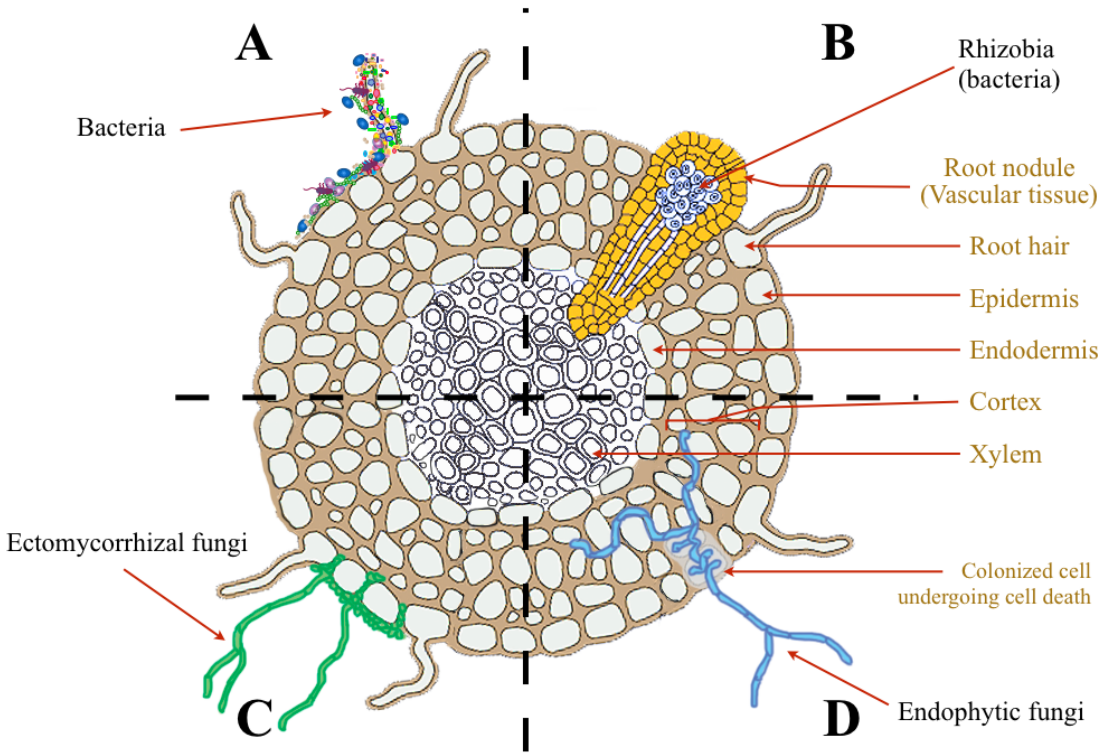


Figure 1-3: Schematic of different ways bacteria or fungi associating with root. Root cross section is presented in brown region, with brown texts indicating different root parts. Black texts indicate bacteria and fungi. **(A).** Bacteria covering plant root. **(B).** Bacteria (usually rhizobia) established in root nodule. **(C).** Fungi extracellularly associating with root. **(D).** Fungi intracellularly connect with root.

Plant beneficial microbes have positive effects on plants in many aspects, one of the key benefits they can provide is to assist plants with nutrient uptake (Yadav *et al.*, 2015). For example, rhizobia fix nitrogen within legume root nodules (Zahran, 1999), and phosphate solubilizing bacteria make organic and inorganic phosphate available to plants via synthesising phosphatases, decreasing soil pH or chelating phosphate with organic acids (Rodríguez and Fraga, 1999; Ordoñez *et al.*, 2016). In addition, siderophore producing bacteria can help plants to acquire iron by secreting siderophores that bind Fe^{3+} and can later been taken up by plants (Richardson *et al.*, 2009). Similarly, arbuscular mycorrhizal fungi (AMF) can assist plant hosts assimilating phosphate via specific phosphate transporters (Karandashov and Bucher,

2005), and contribute to plants nitrogen uptake with mycelium (fungus vegetative part) (Veresoglou *et al.*, 2012). Besides helping plants acquire nutrients, plant beneficial microbes also stimulate plant growth by producing growth regulators. For example, *Azospirillum brasilense* produces the plant hormones cytokinin and indole acetic acid (IAA) to increase the lateral root and root hair development in pearl millet (Tien *et al.*, 1979). They also help plants tolerate abiotic stress factors such as drought, by improving plant water and nutrient uptake with their extraradical hyphal network, changing root architecture (e.g. increasing root branching), and by adjusting solutes K^+, Ca^{2+}, Mg^{2+} or organic acids and sugars in plant hosts (Mendes *et al.*, 2013; Wu *et al.*, 2013). There are also some microbes that have been reported to improve plant immune responses towards other pathogenic microbes. For example, these microbes can activate plant immunity to restrict plant tissue colonisation by pathogens (García-Gutiérrez *et al.*, 2013), cover roots to occupy colonisation sites (Vacheron *et al.*, 2013) and release compounds such as antibiotics to suppress pathogen growth (Qualhato *et al.*, 2013).

Although plant beneficial microbes have positive effects on plants, the interactions among these microbes varies considerably and is not always clear (Deveau *et al.*, 2018). Physical associations between the plant beneficial bacteria and fungi are various: bacteria might adhere to the surface of fungal hyphae (Figure 1-4 A) (Perotto and Bonfante, 1997; Scheublin *et al.*, 2010), live within fungal cells (endosymbiotically Figure 1-4 B) (Perotto and Bonfante, 1997; Bertaux *et al.*, 2003; Sharma *et al.*, 2008) or live freely not associating with fungi and only interfering through metabolites diffusion (Figure 1-4 C) (Toljander *et al.*, 2007).

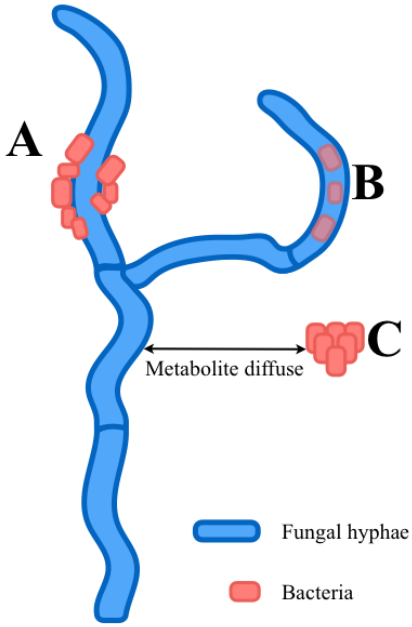


Figure 1-4: Schematic of possible associating ways between bacteria and fungi. **(A).** bacteria adhere to the surface of fungal hyphae. **(B).** bacteria live inside of fungal cell. **(C).** no physical attachment between fungi and bacteria but they are interacting by metabolites diffusion.

There can be many types of interactions among different plant beneficial microbes, and the results can be positive or negative (Ordoñez *et al.*, 2016). For example, there are mycorrhiza helper bacteria like *Bacillus subtilis* that promote the establishment of symbioses between plants and the plant beneficial fungi *Rhizophagus irregularis* (previously called *Glomus. intraradices*) (Toro *et al.*, 1997); with symbiosis plant growth is enhanced. In another study, the presence of plant beneficial fungi *Glomus hoi* causes a decrease in numbers of bacteria belonging to the family Comamonadaceae in the root of plant *Plantago lanceolate* (Nuccio *et al.*, 2013). Interestingly, it was reported that the Comamonadaceae depresses another plant beneficial fungi *Gigaspora rosea* spore germination and hyphal proliferation in the legume *Medicago truncatula* rhizosphere (Pivato *et al.*, 2009).

The interactions among plant beneficial microbes have demonstrated positive effects on the rhizosphere ecology such as promoting plant growth or enhance soil

fertility (Perotto and Bonfante, 1997; Vessey, 2003). Although not fully understood, their synergism could be a key to plant growth. Understanding the dynamics of these interactions can provide significant benefits in future biological and agricultural applications.

1.4 Synthetic microbial communities

Identification and quantification of plant beneficial microbes in rhizospheres is challenging due to their complex biodiversities, rapid changes in metabolism and potential gene transfers (which can equip organisms with new traits) (Philippot *et al.*, 2013; Bulgarelli *et al.*, 2013; Minz *et al.*, 2013). Many studies have attempted to analyse the microbes by profiling on molecular markers with recent advances in metagenomic techniques such as MiSeq sequencing species (Bulgarelli *et al.*, 2013; Bai *et al.*, 2015), and by investigating their biochemical activities using metabolomics approaches such as mass spectrometry and chromatography (Sirrenberg *et al.*, 2007; Nuccio *et al.*, 2013). One advantage of these culture-independent strategies is the ability to describe the physiology and function of microbial communities in fine resolution on metagenomics (genome-wide analysis of DNA obtained directly from the environment), transcriptomics (RNA expression analysis), proteomics (large-scale protein expression analysis), and metabolomics (metabolite profile analysis) (Figure 1-5). However, this omics data mostly provides descriptive information on the composition of microbial communities, and is still limited in the understanding of the metabolic foundation of microbial community interactions (Jessup *et al.*, 2004; Morales and Holben, 2011). One important aspect omics data cannot identify is the spatial organization, which includes, but is not limited to, the distance between adjoining microbial colonies on the root surface or in the rhizosphere soil, or the

layering and patterns of positions of different species and so on. Since much of microbe-microbe communication relies on metabolite diffusion (Peaudecerf *et al.*, 2018), the way in which the microbes associate with each other can determine the degree of interaction between species.

A way to study microbial community without missing the importance of spatial organization is to construct synthetic microbial communities, meaning the design and assembly of new biological systems, or the re-designing of existing or natural biological systems for targeted analyses (De Roy *et al.*, 2014). Such systems can be adjusted to a manageable number of microbe components, thereby enhancing the tractability and understanding of their interactions (Großkopf and Soyer, 2014). Constructing synthetic communities requires the artificial selection of essential organisms which can represent their counterpart in real environments, and the culturing of them in a controllable situation. These artificial systems can serve as a model to study the community under determined functions (e.g. in plant growth promotion) or interaction patterns (like competition, cooperation, etc. as shown in Figure 1-1).

As a build-to-understand approach (Figure 1-5), constructing synthetic microbial communities offers many advantages. For example, controlling and evaluating metabolic interactions can be much more clearly in synthetic communities, the spatial organization can be measurable and controllable, and dynamics over time can be measured in ways that are not possible in a complex community. With these increased measurement abilities, synthetic microbial communities make it possible to test theories that cannot be clarified with a complex community (De Roy *et al.*, 2014). For example, in a designed *E. coli* system containing strains with different amino acid synthesis abilities, costly amino acids (requires more energy to produce) tended to

promote stronger cooperative interactions among organisms than the cheaper ones (Mee *et al.*, 2014). In a mutualistic system containing algal-bacterial co-culture, geometry plays an important role for the exchanging essential metabolites by diffusion (Peaudecerf *et al.*, 2018).

In addition to better understand the fundamental processes regulating community structures and functions, synthetic systems can also have an important role in applications of different fields (De Roy *et al.*, 2014). For example, in constructing closed-cycle ecosystems for space missions, synthetic microbial communities were used to perform individual functions of the life-support system for space crews (Gòdia *et al.*, 2002; Hendrickx *et al.*, 2005). In multi-species bioproduction routes such as wastewater treatment or methane bioproduction, synthetic microbial communities were used to sequentially produce the desired products (Moussa *et al.*, 2005; Schink, 1997).

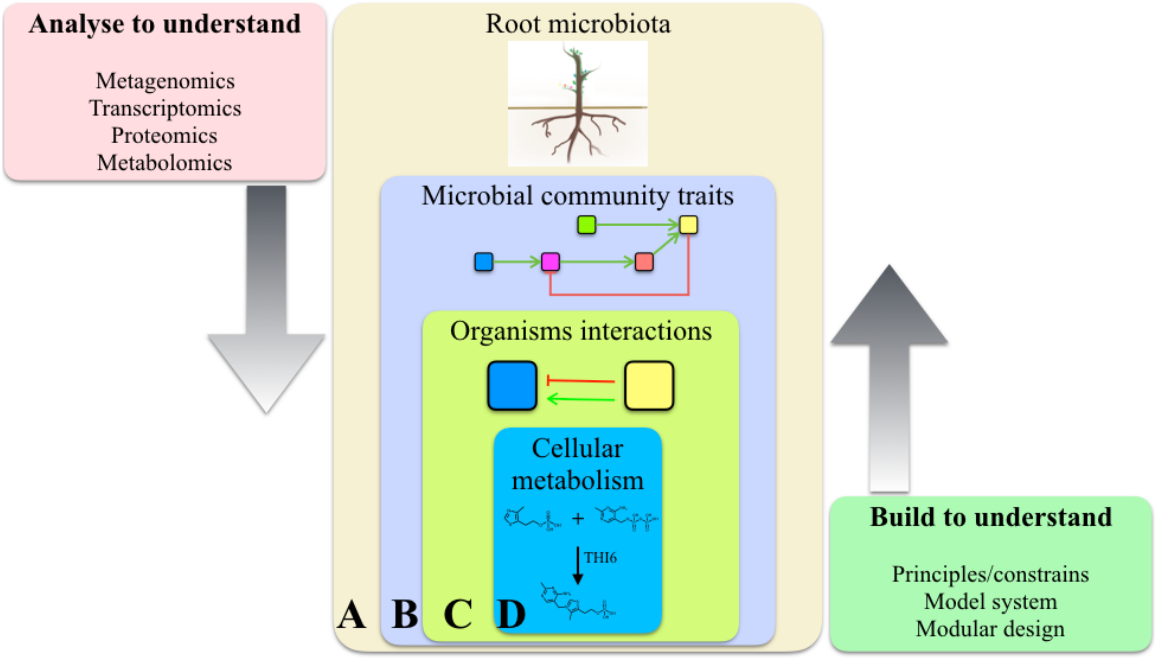


Figure 1-5: Schematic of approaches to understand the microbial communities from two different directions. Pink box on the left indicates the top-down approach of understanding microbial community by analysing the omics data. Green box on the right indicates the bottom-up approach of studying

microbial community by constructing simple organisms and communities. Coloured squares with black line border indicate assorted microbial species. **(A)**, **(B)**, **(C)** and **(D)** indicate elements from different level included in the root microbiota study. **(A).** Root microbiota ecology. **(B).** Microbial community function and dynamics. **(C).** Interactions between two organisms or species. **(D).** Metabolisms within one organism.

1.5 Reductionism as a guiding principle

Synthetic community construction offers a bottom-up, reductionist approach towards understanding the interactions between specific species, and can therefore inform the understanding of more complex systems. As a scientific philosophy, reductionism seeks to explain a complex system by analysing the performances of its constituents (or lower level parts) (Brigandt and Love, 2008). Understanding the smaller parts of a system is crucial for the understanding of a whole system. As such, identifying the presence or loss of certain genes can help in determination of microbes having or lacking certain functions (Fang and Casadevall, 2011).

A map is a general example for reductionism, as it is the representation of geographic data. A satellite photo might be a good representation of the real geography of a location (such as the city London in Figure 1-6 A), but is not straightforward for providing travel information. For travel, a map with labels (Figure 1-6 B) of the roads and places is very informative, even it lacks many information such as landmark appearances when compared to a satellite photo. An underground map (Figure 1-6 C) containing only names of stations and connections in-between can serve a traveller better, as it concisely indicates how to reach a location. The complexity of the underground map is the lowest among the three, but it has kept the vital information for travelling. Even better, its briefness makes the key data stand out

and easy to access. Thus, the reductionist approach (map) can provide a good purpose-oriented (travel) understanding of a system (actual geographic environment).

To understand the root microbiota, a reductionist approach can be applied as follows: the function of root microbiota (Figure 1-5 A) is determined by the present species and the interactions among those species (Figure 1-5 B); the positive effects towards plants come from plant beneficial microbes; the interactions among plant beneficial microbes (Figure 1-5 C) are decided by each organism's metabolism (Figure 1-5 D). By identifying and incorporating the species that are relevant to the plant benefits, related to each other and relatively well-defined, it is possible to create a model system that contains the feature of plant growth promoting, but also tractable for modelling and predicting of interactions under different conditions. Such systems could help with the investigation of the factors affecting the complexity of microbial interactions, and revealing the ways to exploit them for purposes such as enhancing plant growth. Therefore, this study focused on applying the reductionism principle to the construction of synthetic microbial community by composing a simple two-organism system to understand the part of the microbial community it can represents.

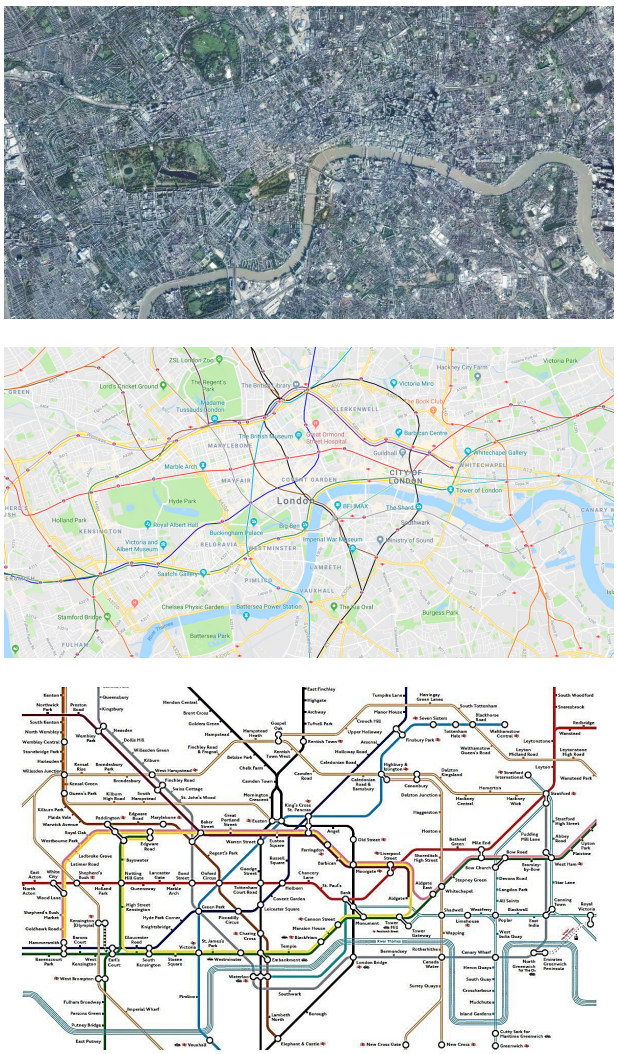


Figure 1-6: Illustration of maps containing different level of complexity, while sharing a same information on transportation. Images are modified from google map **(A** and **B)** and Transport for London website **(C)**.

1.6 Plant beneficial microbes used in this study

The bottom-up approach represents a strategy in which two or more defined microbe species are assembled and functionally analysed in a well-characterised and controlled environment (De Roy *et al.*, 2014). At the stage of choosing the species, a question commonly raised is "how simple is too simple"? The danger of selecting the wrong species or not having enough organisms in the community, might cause a loss of important features of the community, which could result in an inadequate

description or characterisation of the system of interest. On the other hand, any additional organism will level up the complexity of interactions, generating redundant parameters for analysing and results in unnecessary efforts to reach a nonsignificant result.

In this study, a minimal synthetic community was constructed with two plant beneficial microbes: the fungus *Serendipita indica* and the bacterium *Bacillus subtilis*. By using one fungus and one bacterium species, the aim was to represent the two important kingdoms in the rhizosphere community, and to analyse the potential cross-kingdom interactions. Thus, this system could be used to represent organisms from wider range. The choice of those two species is based on the available resources and the functional features of those species explained as follows.

Serendipita indica

S. indica is an endophytic fungus (Figure 1-3 d) that can promote plant growth. It was first discovered in 1998 in the Western Indian Thar Desert (Verma *et al.*, 1998; Varma *et al.*, 1999), and was formerly known as *Piriformospora indica* based on its pear shaped chlamydospores. In 2016, it was reclassified to the genus *Serendipita* and renamed as *Serendipita indica* based on molecular phylogenetic analysis (Weiss *et al.*, 2016) (Table 1-1).

Table 1-1: *S. indica* current classification

MycoBank MB812127	
Division	Basidiomycota
Subdivision	Agaricomycotina
Class	Agaricomycetes
Order	Sebacinales
Family	Serendipitaceae
Genus	*Serendipita*
Species	*Serendipita indica*

S. indica has been well studied for its morphology, metabolism and functions (Singhal *et al.*, 2017a). It can colonise the roots of a wide range of mono- and dicotyledonous plants (Qiang *et al.*, 2012) through the mechanism of root immunity suppression (Schäfer *et al.*, 2009; Jacobs *et al.*, 2011). It can provide a range of beneficial effects to plants, including enhancement of plant growth and resistance to biotic and abiotic stresses (Waller *et al.*, 2005; Sherameti *et al.*, 2008; Baltruschat *et al.*, 2008; Vadassery *et al.*, 2009), stimulation of host plant root branching through auxin production (Sirrenberg *et al.*, 2007), promotion of adventitious root formation in cuttings (Druege *et al.*, 2007), and facilitation of plant phosphate assimilation (Yadav *et al.*, 2010). Unlike many other plant beneficial fungi such as AMF, *S. indica* has the ability to grow saprophytically (obtain nutrient from dead organic matter) in the absence of host plants (Kumar *et al.*, 2011). These features identified *S. indica* as an ideal organism for the initial analyses in this study that aimed at generating a synthetic plant beneficial microbial community.

Bacillus subtilis

B. subtilis is a ubiquitous soil dwelling microbe and a genetically tractable model organism (Kunst *et al.*, 1997; Castillo *et al.*, 2013; Michna *et al.*, 2014). It is found in many soil systems and presented as one of the dominant species (Köberl *et al.*, 2011; Minz *et al.*, 2013). *B. subtilis* has been extensively studied as a plant beneficial microbe from many aspects: as a phosphate solubilizing bacteria (Rodríguez and Fraga, 1999), as a mycorrhizal helper bacteria (Toro *et al.*, 1997), as an antagonist of fungal pathogens (Cazorla *et al.*, 2007) and so on. It is also one of the model organisms for studying thiamine biosynthesis (Jurgenson *et al.*, 2009), which is

an important feature in developing the interactions with *S. indica* (details will be presented in Chapter 3).

Since *B. subtilis* is well studied, using it for constructing a synthetic community could save some efforts in characterising its growth and identifying factors that might affect its metabolisms. The thiamine biosynthesis ability and soil residency make *B. subtilis* functionally and ecologically relevant with *S. indica* and therefore make it ideal for constructing synthetic plant-beneficial microbial community with *S. indica*.

1.7 Project Aims

This study aimed to understand how plant beneficial microbes interact with each other. *Serendipita indica* and *Bacillus subtilis* were used in this project for constructing a minimal synthetic microbial community that can be used as a model system for analysing microbial community interactions. For this purpose, experiments were conducted with a clear focus on

1 Constructing a defined environment for the plant growth promoting fungus *Serendipita indica* (Chapter 2)
2 Constructing a minimal microbial community with a fungal species (*S. indica*) and a bacterial species (*Bacillus subtilis*) and investigating the system dynamics (Chapter 3)
3 Morphological study of this minimal synthetic community and developing microscopy observation method (Chapter 4)

Chapter 2

Thiamine auxotrophy of *Serendipita indica*

The following parts of this chapter are included in the author's publication (Jiang, X. *et al.*, 2018): section 2.4 (except figure 2-9) and parts of section 2.3 and 2.5.

2.1 Abstract

The plant beneficial fungus *Serendipita indica* is cultivable without association with a plant host. Using *S. indica* to construct a synthetic microbial community for a better understanding of microbial community, a defined medium is required as a controllable environment. To achieve this goal, a well-defined medium that can support *S. indica* growth was constructed. An auxotrophy in *S. indica* for thiamine, a key co-factor for essential reactions in the central carbon metabolism, was identified. The genomic enzyme contents on thiamine auxotrophy of *S. indica* were analysed to understand its metabolism relating to thiamine. Phylogenetic and bioinformatic analyses were performed on fungi close to *S. indica* for understanding the distribution of thiamine biosynthesis abilities. The developed synthetic medium creates a defined environment for studying *S. indica*. The discovery made in this study is the first report on *S. indica* thiamine auxotrophy.

2.2 Introduction

Serendipita indica is a root endophytic fungus that can colonise a broad spectrum of plants and can provide growth promoting effects to the plant hosts (Qiang *et al.*, 2011). Unlike many other mycorrhizal fungi, it can be cultivated without the presence of live plant hosts (Pham *et al.*, 2008). This makes *S. indica* easy to cultivate and allows for analysis without interference from plants. Based on these features, this study uses *S. indica* as a representative for plant beneficial fungi in a synthetic microbial community, for the bottom-up approach of studying microbial community.

Cultivation methods are already established, and various media recipes reported for *S. indica* (Zuccaro *et al.*, 2011; Kumar *et al.*, 2011; Jacobs *et al.*, 2011; Varma *et al.*, 2012; Qiang *et al.*, 2011). However, these commonly used media recipes for *S. indica* are not ideal for the purpose of this study in creating a well-defined environment. There are always some ingredients of undefined composition in these media, such as yeast extract, malt extract and peptone used in Hill and Kaefer medium (Kumar *et al.*, 2011; Singhal *et al.*, 2017a), aspergillus broth medium (Pham *et al.*, 2008) or complete medium (Zuccaro *et al.*, 2011). These undefined ingredients are replaced by a set of vitamins in other media such as the yeast nitrogen base medium (Zuccaro *et al.*, 2011) to support *S. indica* growth. Removing these undefined factors or vitamin mixtures will make these media unable to maintain *S. indica* growth.

Although the undefined factors in media vary among recipes, the macronutrients such as carbon and nitrogen source *S. indica* requires are well-studied. Knowing these nutrient requirements will help in designing synthetic medium with controlled diversities. Several carbon sources such as glucose, maltose, xylose, sucrose, lactose, rhamnose and arabinose, have been tested and all generate good growth of *S. indica* (Pham *et al.*, 2008). For the nitrogen source, *S. indica* grows best on ammonium or glutamine, and cannot utilise nitrate due to the lack of nitrate transporter (Zuccaro *et al.*, 2011). On the phosphate aspect, *S. indica* can utilise a variety of inorganic and organic phosphate sources.

On the other hand, it is important to know the basic morphology of *S. indica* when setting the cultivation methods. The structure of *S. indica* consists of hyphae and chlamydospores (Figure 2-1). The hyphae of *S. indica* are strings of beads-like cells. No sexual structures or special connections like clamp were reported for *S.*

indica (Varma *et al.*, 1999; Singhal *et al.*, 2017b). The chlamydospores (dormant and asexual spores) of *S. indica* are reported to have autofluorescence (Pham *et al.*, 2008; Singhal *et al.*, 2017b), which can be caused by the accumulation of NAD(P)H in spores (Siddhanta *et al.*, 2017; Singhal *et al.*, 2017b). On solid medium (Figure 2-2 A), *S. indica* expands concentrically and presents a round colony on the medium surface, and forms rhythmic rings as the colony expands (Varma *et al.*, 1999; Singhal *et al.*, 2017b). In liquid medium (Figure 2-2 B), hyphae and spores of *S. indica* cluster into rod-shaped balls with fuzzy surface and irregular size. The colour of *S. indica* colony is white to pale yellow (Pham *et al.*, 2008). These morphological features could serve as references in identifying *S. indica* and evaluating its growth status in newly designed cultivation conditions.

The work in this chapter began with the identification of a defined medium for *S. indica* growth. In doing so, the fact was discovered that *S. indica* is auxotrophic for a specific vitamin, thiamine. This thiamine auxotrophy for *S. indica* is reported for the first time. To support this physiological finding, bioinformatic analysis was performed on *S. indica*, which allowed me to identify the key biosynthesis genes missing from its genome. Finally, these findings were put in a broader context by performing a phylogenetic analysis of the distribution of these key genes in different fungi. These findings allowed the establishing of defined growth conditions for *S. indica*, as well as the exploring its thiamine auxotrophy as a mechanism to establish co-cultures with bacteria (as described in Chapter 3).

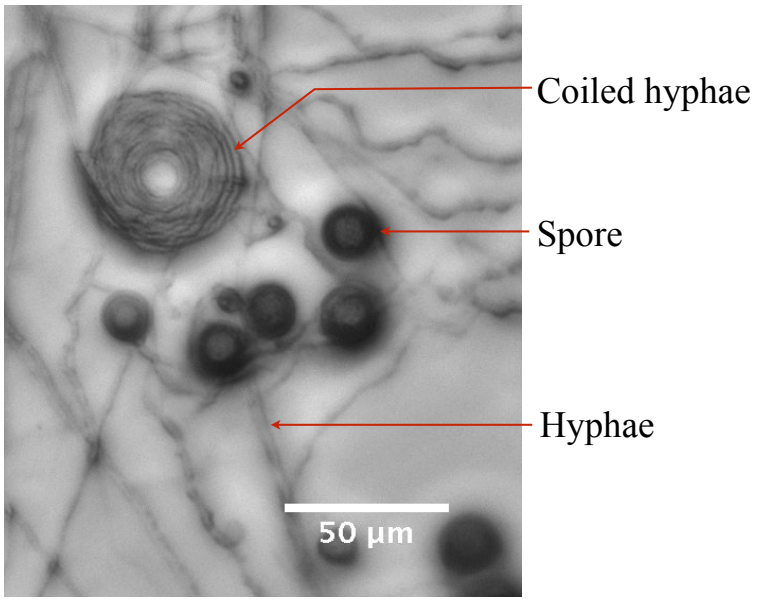


Figure 2-1: *S. indica* structures under the microscope. Image was taken on 1 week after inoculation in liquid synthetic medium containing thiamine.

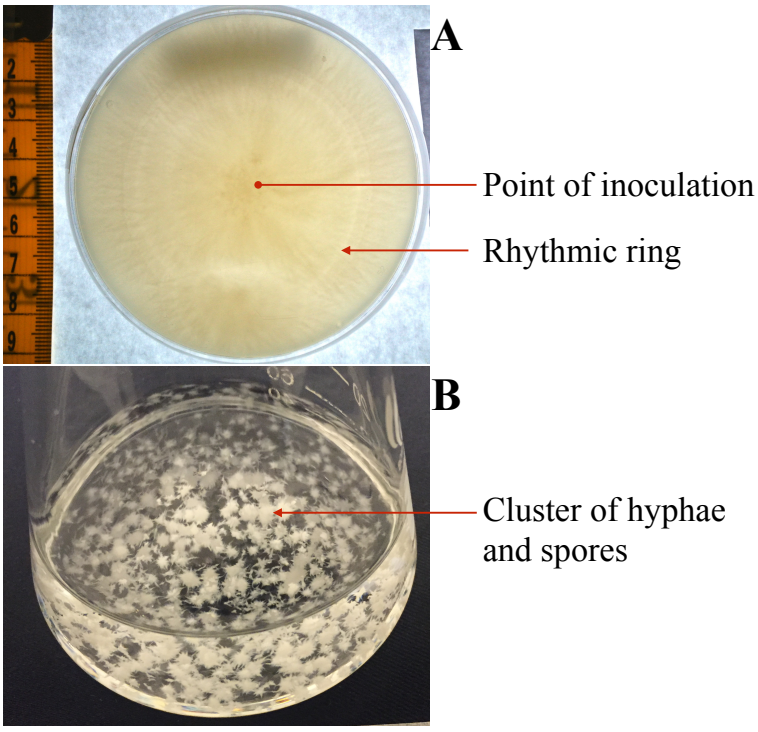


Figure 2-2: *S. indica* cultivated on agar plate **(A)** and in liquid culture **(B)**. Images were taken on 6 weeks after inoculation on agar synthetic medium containing thiamine, and on 1 week after inoculation in liquid synthetic medium containing thiamine.

2.3 Materials and methods

Analytical-grade chemicals were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA), or Fisher Scientific UK (Loughborough, UK).

In this chapter, the basic synthetic medium is defined as the one containing macroelements and microelements indicated in table 2-1, without adding any vitamin or undefined elements such as thiamine or yeast extract. Additional elements such as thiamine, agar or bromocresol purple listed in (Table 2-2) was for reference. The inclusion of these elements is stated in each experiment description.

2.3.1 Synthetic medium for cultivating *S. indica*

This synthetic medium was designed based on a combination of reported *S. indica* media (Zuccaro *et al.*, 2011; Kumar *et al.*, 2011; Jacobs *et al.*, 2011; Varma *et al.*, 2012; Qiang *et al.*, 2011) and a bacteria medium reported in MELiSSA project (Montràs *et al.*, 2008).

Each liter of the basic synthetic medium contains 20 g glucose, 1.32 g $(NH_4)_2SO_4$, 0.89 g $Na_2HPO_4 \cdot 2H_2O$, 0.68 g KH_2PO_4, 35 mg $Na_2MoO_7 \cdot 2H_2O$, 5.2 mg $MgSO_4 \cdot 7H_2O$, 2.5 mg $FeSO_4 \cdot 7H_2O$, 0.74 mg $CaCl_2 \cdot 2H_2O$, 0. 0043 mg $ZnSO_4 \cdot 7H_2O$, 0. 004 mg $CuSO_4 \cdot 5H_2O$. The 20 mM nitrogen from $(NH_4)_2SO_4$ source is replaced with 1.46 g glutamine for a similar growth effect or replaced with 1.7g $NaNO_3$ for negative effect (no growth) in comparison, as stated in each experiment description. In previous report on *S. indica* growing on defined medium, ammonium and glutamine generate similar growth effect to *S. indica*, while nitrate is not usable to *S. indica* due to its lacking nitrate transporter (Zuccaro *et al.*, 2011).

Table 2-1: Synthetic medium for *S. indica*

Medium composition	**Chemical name**	**Molar concentration**	**Weight in 1 L**
Buffer system	$Na_2HPO_4 \cdot 2H_2O$	5 mM	0.89 g
	KH_2PO_4	5 mM	0.68 g
Salts and micro-elements	$Na_2MoO_4 \cdot 2H_2O$	145 µM	0.035 g/ 3.5% (w/v) stock: 1ml
	$MgSO_4 \cdot 7H_2O$	211 µM	0.0052g/ 5.2% (w/v) stock: 1ml
	$FeSO_4 \cdot 7H_2O$	9 µM	0.0025 g/ 0.25% (w/v) stock: 1ml
	$CaCl_2 \cdot 2H_2O$	5 µM	0.00074 g/ 0.74% (w/v) stock:100 µl
	$ZnSO_4 \cdot 7H_2O$	0.15 µM	0. 0043 mg/ 0.0043% (w/v) stock: 100 µl
	$CuSO_4 \cdot 5H_2O$	0.16 µM	0. 004 mg/ 0.004% (w/v) stock: 100 µl
Carbon source	Glucose	111 mM	20 g
Nitrogen source (only one of them)	Glutamine	20 mM of Nitrogen	1.46 g
	$(NH_4)_2SO_4$		1.32 g
	$NaNO_3$		1.7 g
Vitamin	Thiamine (hydrochloride)	0.5 µM	150 µg/l 100 mg/l stock: 150 µl per 100 ml medium
pH indicator	Bromocresol purple	37 µM	0.02 g/l 2% (w/v) stock (80% ethanol as solution): 0.1 ml per 100ml medium
Solidifying agent	agar		15 g

For the preparation of 1 liter liquid medium, all the components were added together and brought to the volume of approximate 900ml with MilliQ water. The pH was adjusted to 7 with approximate 1ml of 1 M HCl. Then the volume was filled up to 1 liter. Because glucose caramelised at pH7 after autoclaving, the liquid medium was instead filter sterilised using sterile 0.2 µm nylon filter (Ref: Z290823, Sigma).

For the preparation of agar medium, to avoid glucose caramelisation, a two times concentrated liquid medium was prepared, and filter sterilised as mentioned above. A 3% (weight/volume) agar solution was prepared with MilliQ water then autoclaved.

The final 1.5% agar medium were made by mixing the double-concentrated liquid medium with the same volume as the 3% agar solution.

In experiments testing the pH changes of *S. indica* growth, pH indicator bromocresol purple was used. The bromocresol purple was pre-dissolved in 80% ethanol and added to liquid medium before sterilisation.

For cultivating *S. indica* in the synthetic medium, thiamine was used for supporting positive *S. indica* growth. Solution of thiamine-HCl was pre-filter sterilised and added after sterilisation to the final medium when the temperature was below 40℃. The reason for using thiamine will be explained in result section 2.4.1.

2.3.2 *S. indica* spore suspension preparation

This method is adopted from Dr. Patrick Schäfer lab (Reitz *et al.*, 2012). Materials and equipment including tween water (200 µl tween 20 in 1 liter MilliQ water), miracloth (Ref: 475855, Merck Millipore), glass funnel, forceps and Drigalski spatula were autoclaved before use. Healthy *S. indica* (no abnormal growth or getting contamination) growing on complete medium 1.5% (w/v) agar plates (Pontecorvo *et al.*, 1953; Zuccaro *et al.*, 2011) (Table 2-2) for 4~6 weeks old were selected for spore suspension preparation. At least two plates were used for each experiment to minimise the growth variance from the individual plate. Each plate was poured with approximate 50ml tween water and scrubbed with a spatula, so that spores and hyphae could be washed off and be transferred into a sterile flask or tube (conical polypropylene centrifuge tube, Ref 352070, Falcon) (Figure 2-3). The flask was shaken vigorously then sonicated for 3-5 mins. Afterwards, spore suspension was filtered into sterile centrifuge tubes using 2 layers of miracloth. The tubes were centrifuged for 5-7 mins at room temperature and 2000 g. Afterwards, the supernatant

was discarded, and the spores were suspended with 40 ml tween water. This washing step was repeated 3 times before a final sonication of 5 min.

Spore suspension concentration was then determined using a Fuchs-Rosenthal haemocytometer. On each side of the haemocytometer one large square (16 small squares) plus the diagonal 4 small squares of another (20 small squares altogether) were counted. The mean of the two values was taken and multiplied by 4,000 to calculate the number of spores per ml (this is based on the user guide of haemocytometer). The spore suspension was adjusted with sterile tween water to the final concentration of 500,000 per ml.

Table 2-2: Complete medium for *S. indica*

CM (complete medium)	1 L
Glucose	20 g
Peptone from casein	2 g
Yeast extract	1 g
Casamino acids	1 g
Agar	15 g
20X Salt solution	50 ml
1000X Microelements	1 ml
20X Salt solution	1 L
$NaNO_3$	120 g
KCl	10.4 g
$MgSO_4$ x $7H_2O$	10.4 g
KH_2PO_4	30.4 g
1000X microelements	1 L
$MnCl_2$ x $4H_2O$	6 g
H_3BO_3	1.5 g
$ZnSO_4$ x $7H_2O$	2.65 g
KI	750 mg
Na_2MO_4 x $2H_2O$	2.4 mg
$CuSO_4$ x $5H_2O$	130 mg

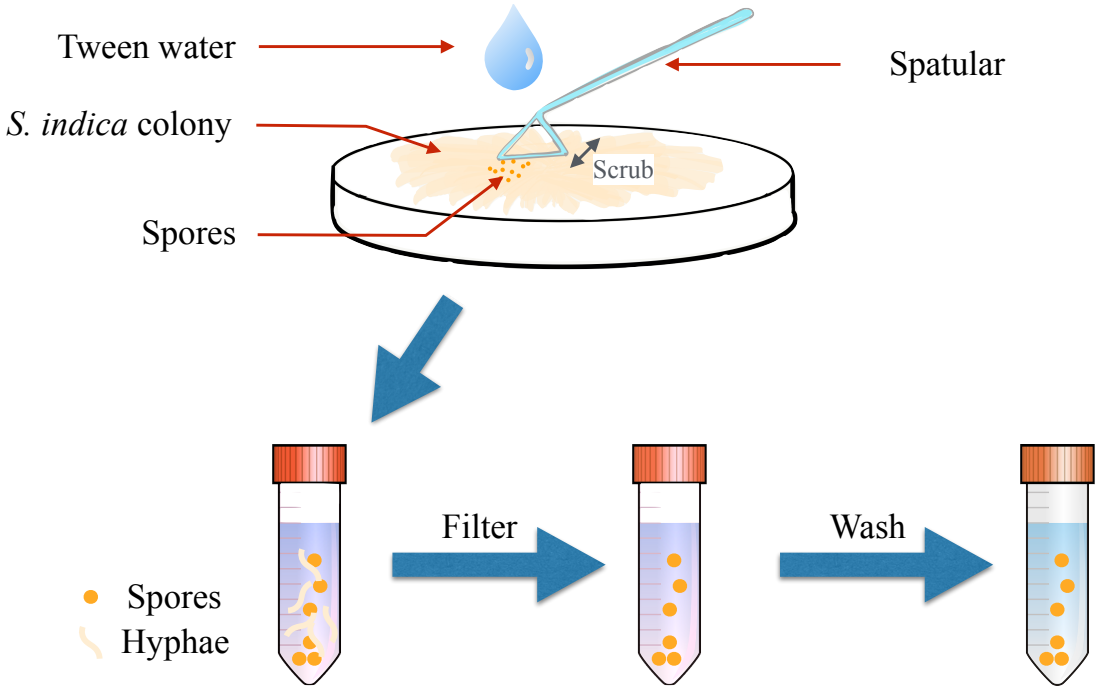


Figure 2-3: Schematic of *S. indica* spore suspension preparation. Detailed steps are described in section 2.3.2. *S. indica* colony on CM plates is washed with tween water and scrubbed with spatula. The washed off liquid contains spores and hyphae. Hyphae are filtered out. The filtered spore suspension is then washed with tween water.

2.3.3 *S. indica* growth condition screen

The 24-well plates (Ref: 3047, Corning) and the basic synthetic medium containing ammonium as the sole nitrogen source were used for performing the growth condition screen for *S. indica*.

In the vitamin screen, each well contained 1ml of basic synthetic agar medium. In each of the sole-vitamin treatment, the final concentration of each added vitamin was 200 µg/l. In the vitamin-mixture treatment, the tested vitamins were added together into the basic medium with a final concentration of 200 µg/l of each vitamin. The yeast extract treatment containing 1 g/l yeast extract served as a positive control. The negative control contained only the basic medium without any additional elements.

In the organic acid and amino acid screen, 1 ml of basic synthetic agar medium containing one amino acid or organic acid of 5 mM final concentration were used for each single treatment. In the mixture treatments, 1 mM of each element was combined. The yeast extract treatment containing 1 g/l yeast extract served as a positive control. The negative control contained only the basic medium without any additional element.

S. indica spore suspension were inoculated 1 µl onto the centre of each well. In one of the negative controls, 1 µl of tween water was inoculated instead of spore suspension. Each treatment was prepared in three technical replicates. 24-well plates were then sealed with parafilm and incubated statically at 30℃ for 2 weeks. Images were taken with a gel doc system (G:Box EF, Syngene).

2.3.4 *S. indica* interaction with thiamine agar block

On each of the 60 mm petri dishes (Ref: 1007, Corning), there were 6 ml of basic synthetic agar media containing different nitrogen sources (ammonium or glutamine) and thiamine conditions (with/without thiamine). 2 µl *S. indica* spore suspension was inoculated onto the left side of each plate, while a thiamine block was placed around 2 cm to the right of the inoculum on each treatment accordingly (Figure 2-4). The thiamine block was prepared by punching a thiamine agar plate using the wide end of a 200 µl pipette tip. The thiamine agar plate was made from 60 mm petri dish containing 6 ml of 150 µg/l thiamine-HCl in 1.5% agar. Each thiamine block contained approximately 0.0056 µg Thiamine-HCl based on calculation. Plates were sealed with parafilm and incubated statically at 30 ℃ for 2 weeks. Images were taken with a gel doc system (G:Box EF, Syngene).

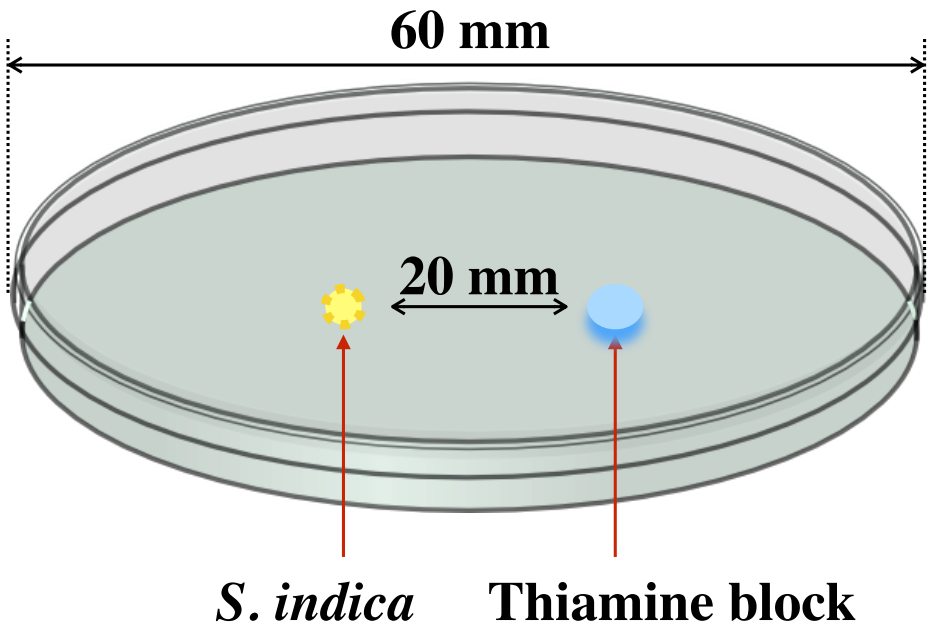


Figure 2-4: Inoculation scheme of *S. indica* and thiamine block on an agar plate. *S. indica* inoculum is shown in the yellow circle; thiamine block is shown in the blue circle. The diameter of the plate is 60 mm. There is a 20 mm space in between *S. indica* and thiamine block.

2.3.5 *S. indica* growth on different thiamine concentrations and nitrogen sources

Medium containing final thiamine concentrations of 0 µg/l, 1.5 µg/l, 15 µg/l, 150 µg/l and 1,500 µg/l were prepared respectively, and distributed in 6-well plates (Ref: 353046, Falcon). Each 6-well plate contained one thiamine concentration condition, with 3 ml medium in each well. On each plate, 1 µl of *S. indica* (500,000 spores/ml) were inoculated in the centre of 5 wells (so that each treatment had 5 replicates), while one well was intentionally left non-inoculated as a blank for plate reader reading (Figure 2-5). Plates were incubated at 30 ℃ for 2 weeks. Afterwards, lids were removed, and OD_{600} and spore fluorescence (at 390 nm for excitation and 470 nm for emission (Siddhanta *et al.*, 2017)) were measured using a plate reader

(CLARIOstar, BMG Labtech), with its plate scan function to get an overall reading of each well. Images of plates were taken using gel doc system (G:Box EF, Syngene).

The same procedure was conducted for testing *S. indica* growing on different thiamine concentrations and nitrogen sources. Medium containing ammonium, glutamine and nitrate as the sole nitrogen source, with final thiamine concentrations of 0 µg/l, 1.5 µg/l, 15 µg/l, 150 µg/l were prepared respectively. All the other steps were the same as described above.

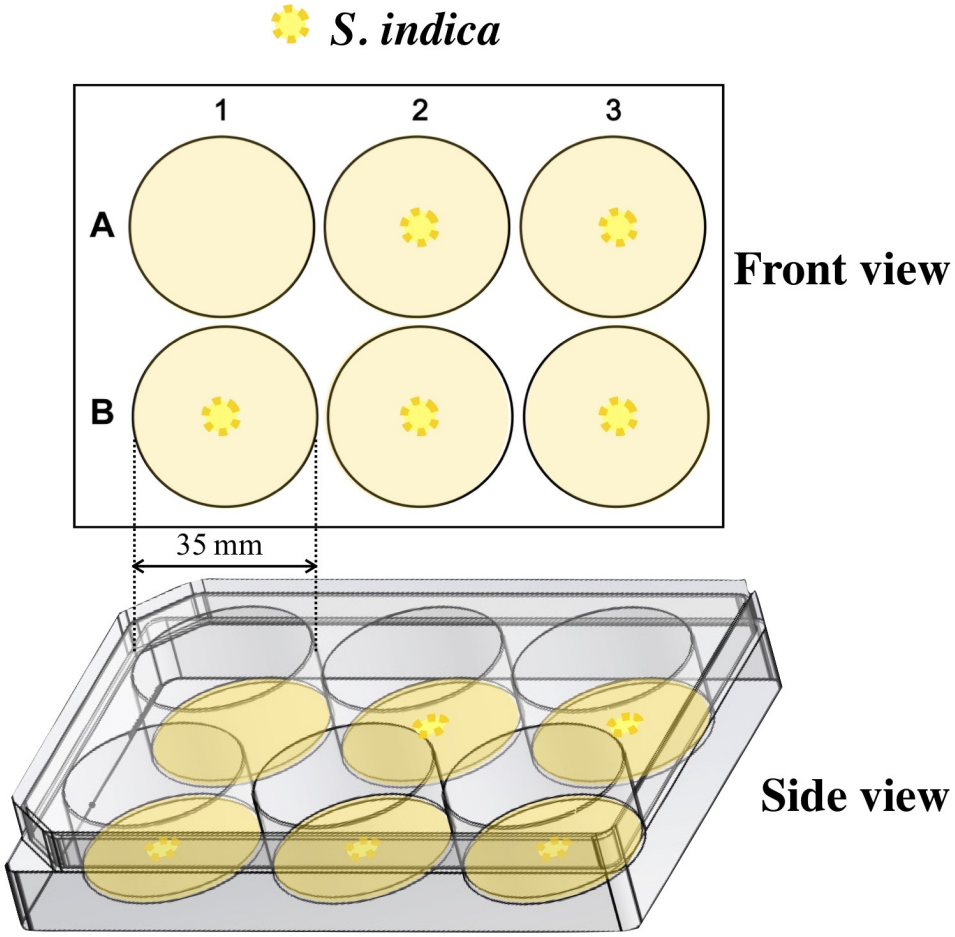


Figure 2-5: Inoculation scheme of *S. indica* on 6-well plate for growth test using plate reader. The A1 position on the plate containing medium is intentionally left un-inoculated for serving as a blank for plate reader reading. The diameter of each well is 35 mm.

2.3.6 Sequence analysis and BLAST search of key thiamine biosynthesis genes

To understand how *S. indica* acquires and processes thiamine, genetic analysis was done to search for thiamine related genes. *Saccharomyces cerevisiae* is one of the model organisms used to study the thiamine biosynthesis pathway (Hohmann and Meacock, 1998). Gene sequences of key thiamine biosynthesis enzymes (*THI6*, *THI20*, *THI4*, *THI5*, *THI80*) and thiamine salvage pathway related proteins (*PHO3*, *THI7*) from *Saccharomyces cerevisiae* S288c were compared with available *S. indica* genome homologues using Position-Specific Initiated BLAST, to identify the putative functions of the corresponding genes (https://blast.ncbi.nlm.nih.gov/Blast.cgi). An e-value of $1e^{-6}$ was chosen as cut-off to identify homologous sequences (Altschul, 1997).

2.3.7 Phylogenetic and bioinformatics analyses

A phylogenetic analysis was performed on 162 fungi species including *S. indica* and its close relative *S. vermifera*, and focused on thiamine biosynthesis genes (*THI6*, *THI20*, *THI4*, *THI5*, *THI80*). This focus meant that only species with fully-sequenced and well-annotated genomes can be used. To collect fungi species fulfilling these criteria, the KEGG database (Kanehisa and Goto, 2000) was used, in which there are 102 species with annotated genomes that are mapped to metabolic pathways. This dataset, however, contains only 16 species in the Agaricomycetes class, in which the species *S. indica* and *S. vermifera* belong. To get information for more close relatives of *S. indica*, the NCBI Taxonomy (Federhen, 2012) and SILVA rRNA (Quast *et al.*, 2013) databases were used for collating 60 species that are from the Agaricomycetes class and that have full genome assembly information. For the resulting 162 species, their SSU-rRNA (small subunit ribosomal RNA) sequences were downloaded from SILVA rRNA database (Quast *et al.*, 2013). Where multiple sequences were

presented for a given species, the longest sequence with highest quality was used. Then the resulting 162 rRNA sequences were aligned using the MUSCLE tool (Edgar, 2004), and a maximum likelihood phylogenetic tree was built using PhyML (Guindon *et al.*, 2010). Onto the resulting phylogenetic tree, the presence and absence information of the thiamine biosynthesis genes *THI6*, *THI20*, *THI5*, *THI4* and *THI80* was mapped. This information was already available for the 102 species, that are contained in the KEGG database. To obtain it for the additional 60 species from the Agaricomycetes class (including the species *S. indica* and *S. vermifera*), BLAST analysis was run on each of these species, with querying each of these species genomes against the *THI6*, *THI20*, *THI5*, *THI4* and *THI80* gene sequences from *S. cerevisiae*. A gene was considered present in a given genome if there was a match of homologous sequences with an e-value smaller than $1e^{-6}$ (Altschul, 1997).

2.4 Results

2.4.1 *S. indica* is auxotrophic for thiamine.

S. indica is an endophytic fungus that can colonise roots of a wide range of plants and can confer a range of beneficial effects, including enhancing plant growth, resistance to biotic and abiotic stresses (Waller *et al.*, 2005; Sherameti *et al.*, 2008; Vadassery *et al.*, 2009), promotion of adventitious root formation in cuttings (Druege *et al.*, 2007), and assisting phosphate assimilation (Yadav *et al.*, 2010). Despite its broad host range, *S. indica* also can grow in the absence of host plants (Kumar *et al.*, 2011). Exploiting this ability, attempts have been made to create a fully defined growth medium that was based on previous physiological studies on *S. indica* (Zuccaro *et al.*, 2011; Kumar *et al.*, 2011; Jacobs *et al.*, 2011; Qiang *et al.*, 2011; Varma *et al.*, 2012).

Using the defined basic synthetic media, the effect of the vitamins on *S. indica* growth was tested by cultivating *S. indica* in a series of vitamin-free media each supplemented by a specific vitamin (Figure 2-6). These tested vitamins were from the list of the media recipes used for *S. indica* cultivation. The results showed that *S. indica* is auxotrophic for thiamine; while none of the other individual vitamin additions supported growth, thiamine and full vitamin addition did.

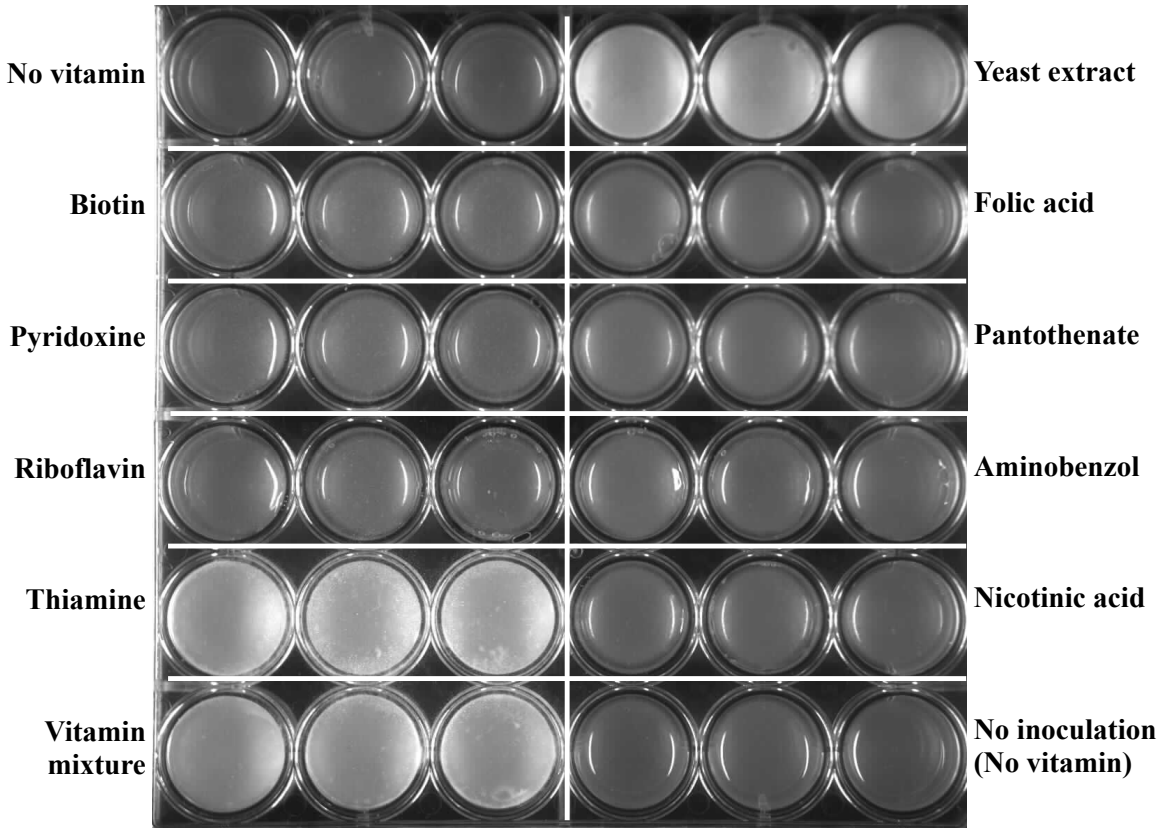


Figure 2-6: Vitamin screen on *S. indica* growth. Two weeks' *S. indica* growth on agar plates with defined medium supplemented with different vitamins as shown on each row and column. Each treatment has three replicates presented in 3 adjacent wells. The diameter of each well is 16.5 mm. *S. indica* growth is visible as white colonies on the surface of a well. Treatments with yeast extract and vitamin mixture served as positive controls. Treatments with no vitamin and no inoculation (no vitatime) served as negative controls. Images were taken after two weeks of growth. Two biological replicates of this experiment were performed with qualitatively similar results.

2.4.2 Testing *S. indica* thiamine dependency with different nitrogen sources

Thiamine necessity to *S. indica* was further confirmed by growing *S. indica* on plates containing different nitrogen sources and supplemented with an additional agar block only containing a defined amount of thiamine. In this case, the growth of *S. indica* resulted in expansion towards the thiamine agar block (Figure 2-7), suggesting that growth occurs on a thiamine gradient or is linked with active chemotaxis towards the thiamine source.

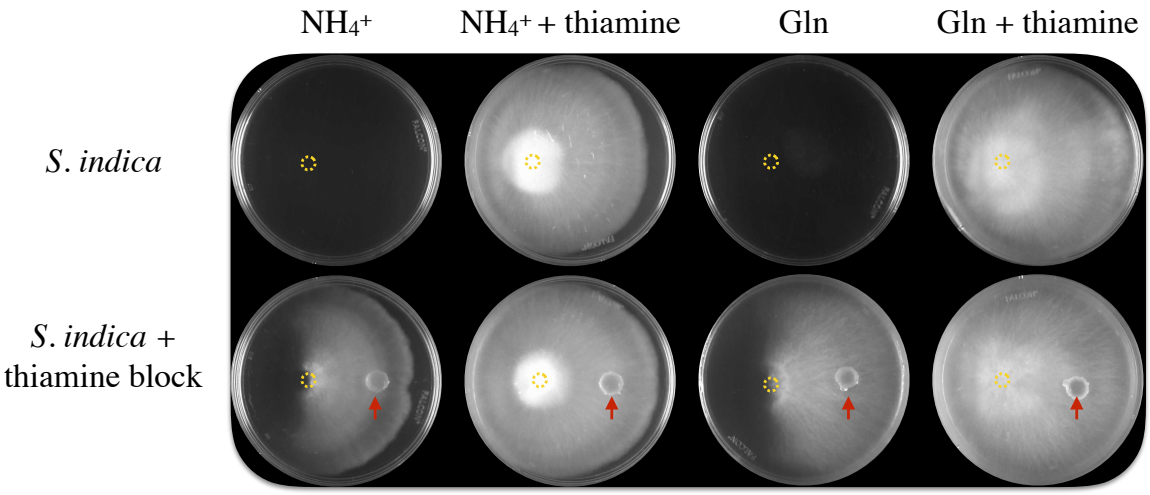


Figure 2-7: *S. indica* growth test with directional thiamine source. Columns show different media containing thiamine or not, and using ammonium and glutamine as nitrogen sources (shown as "NH_4^+" or "Gln" on the panels). The top and bottom rows show the images of agar plates without or with an additional agar block containing thiamine (pointed by red arrow), placed ~1.5 cm to the right side of *S. indica* inoculation point (indicated with a yellow circle). *S. indica* growth is visible as white colonies on the surface of a plate. *S. indica* monocultures on ammonium and glutamine media are negative controls. *S. indica* monocultures on ammonium and glutamine media containing thiamine are positive controls. The diameter of each plate is 60 mm. Images show two weeks' growth of *S. indica*. Each treatment was repeated 3 times, and images shown here are representatives for each treatment. Two biological replicates of this experiment were performed with qualitatively similar results.

The growth of *S. indica* was also quantified with different concentrations of thiamine. The hyphae growth and spore formation showed a positive, but saturating, dependency on thiamine levels in media (Figure 2-8). At zero concentration of thiamine in the media, germination and very little hyphal growth was still observed, possibly supported by spore-stored thiamine.

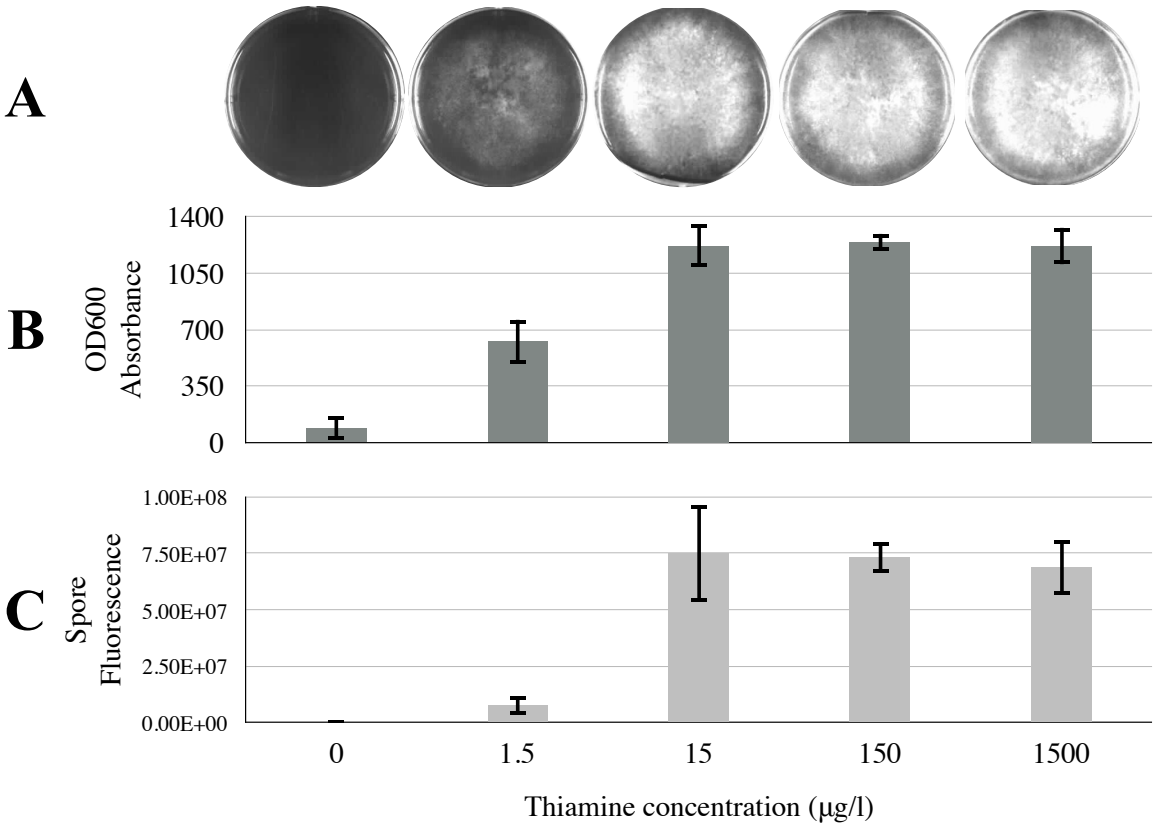


Figure 2-8: *S. indica* growth on medium containing different concentrations of thiamine and ammonium as the nitrogen source. **(A)**. Images show two weeks' growth of *S. indica* on agar plates, and at different concentrations of thiamine as shown below on the bottom x-axis. *S. indica* growth is visible as white colonies on the surface of a plate. The diameter of each image is 35 mm. Plates shown are representative of at least 5 replicates for each condition. Each treatment was repeated 6 times, and images shown here are the representatives of each treatment. **(B)** and **(C)** bar-plots show plate absorbance (at OD_{600}) and fluorescence intensity (measured at 390 nm excitation and 470 nm emission for detection of *S. indica* spores) respectively. Optical density at 600nm in **(B)** indicates the overall growth of *S. indica* colony on agar in each treatment. Spore fluorescence in **(C)** reveals spore quantity that linked to *S. indica* reproductivity. Plate reader (CLARIOstar, BMG Labtech) was used for measuring OD_{600} and fluorescence intensity. Five technical replications were used for generating the intensity data and standard deviation error bars. Another biological replicate (with one less thiamine concentration treatment) was performed with qualitatively similar results and is included in figure 2-9.

This thiamine dependency on growth of *S. indica* has also been tested in combination with different nitrogen sources. In media containing glutamine and

ammonium as nitrogen sources, *S. indica* hyphae growth and spore formation showed positive correlation with thiamine concentration. While in medium containing nitrate as the sole nitrogen source, no spore formation was detected, and very little hyphae growth was detected only at highest tested thiamine concentration of 150 µg/l (Figure 2-9). Since *S. indica* cannot utilise nitrate (Zuccaro *et al.*, 2011), this little growth could possibly be supported by nitrogen element from thiamine or by spore-stored thiamine.

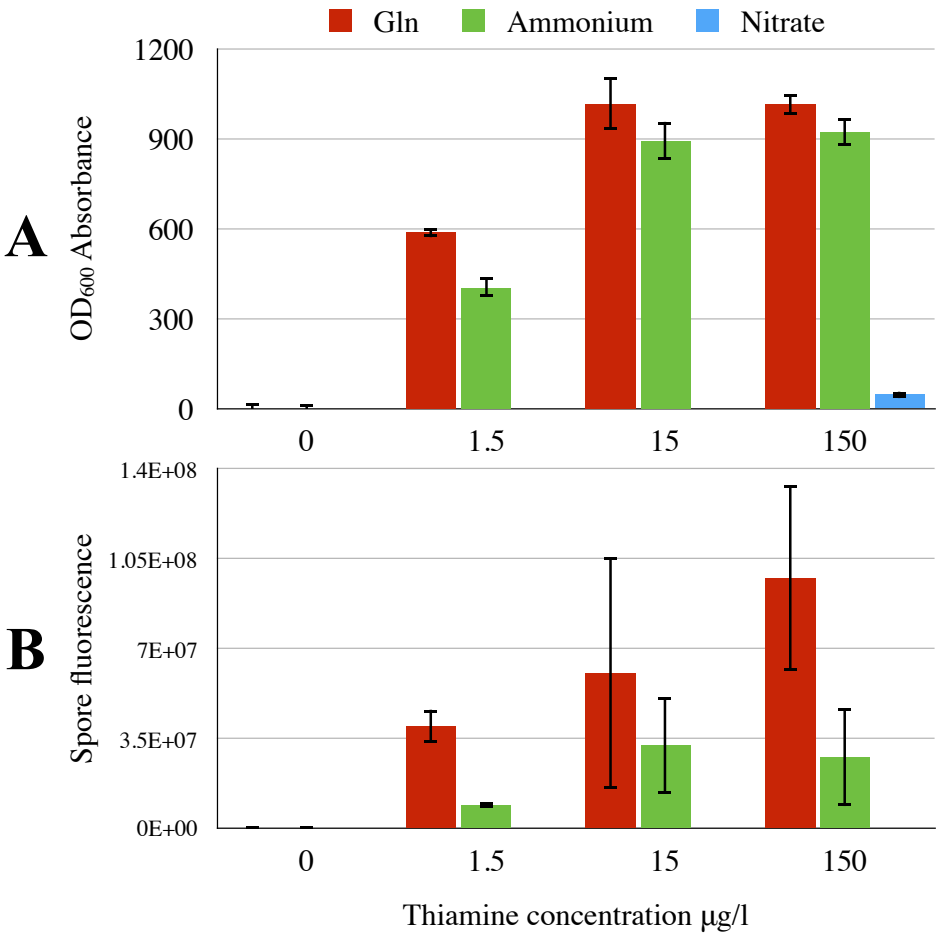


Figure 2-9: *S. indica* growth on media containing different concentrations of thiamine and different nitrogen sources. *S. indica* grew on agar plates for two weeks at different concentrations of thiamine (as shown below on the bottom x-axis) and different nitrogen source (red bars for glutamine, green bars for ammonium and blue bars for nitrate). **(A)** and **(B)** bar-plots show plate absorbance at OD_{600} and fluorescence intensity (measured at 390 nm excitation and 470 nm emission for detection of *S. indica* spores) respectively. Optical density at 600nm in **(A)** indicates the overall growth of *S. indica* colony

on agar in each treatment. Spore fluorescence in **(B)** reveals spore quantity that linked to *S. indica* reproductivity. Plate reader (CLARIOstar, BMG Labtech) was used for measuring OD_{600} and fluorescence intensity. Five technical replications were used for generating the intensity data and standard deviation error bars. There is no biological replicate on glutamine and nitrate treatment due to time limit of the project.

2.4.3 Thiamine auxotrophy cannot be bypassed by organic and amino acid provision

Thiamine is a very important factor for many enzymes in central carbon metabolism (Helliwell *et al.*, 2013). The active form of thiamine in organisms is thiamine pyrophosphate (ThPP), a key co-factor involved in central metabolic reactions (Kraft and Angert, 2017). In particular, ThPP is involved in pyruvate fermentation and conversion for entry into the citric acid cycle (TCA), α-ketoglutarate to succinyl-CoA conversion in the TCA cycle (Figures 2-10 and Figure 2-11), transketolase reactions in the pentose phosphate pathway, and biosynthesis reactions for leucine, isoleucine and valine (Michal and Schomburg, 1999).

To understand if there is any other metabolite that might help *S. indica* bypassing of central reactions requiring thiamine, an analysis has been done on the growth of *S. indica* in the absence of thiamine but supplemented with organic and amino acids that link to the central carbon metabolism (experiment details can be found in section 2.3.3). None of the 17 amino acids or 8 organic acids tested, or their combinations allowed for *S. indica* growth in the absence of thiamine (Figure 2-12). This finding further confirmed that *S. indica* growth is linked directly to thiamine.

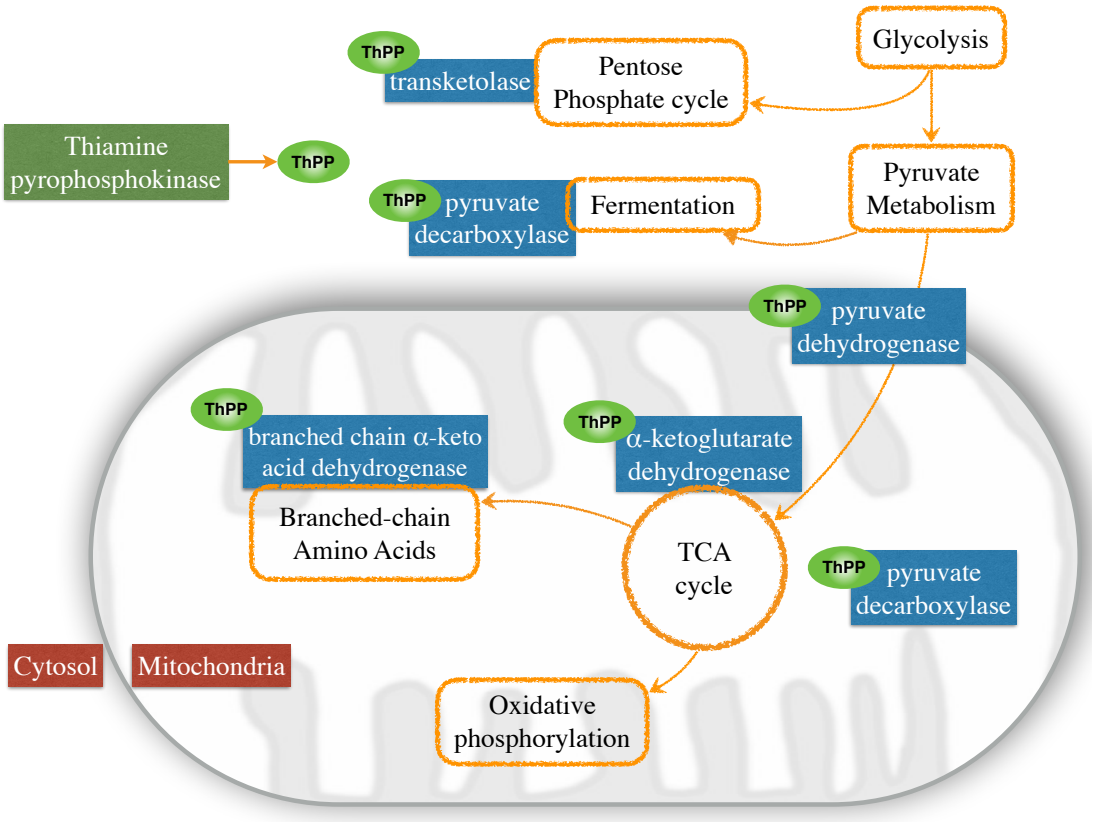


Figure 2-10: Simplified schematic of central metabolism in eukaryotic cells with cytosol and mitochondria compartments indicated. Orange enclosures show reactions of the central metabolism, while blue boxes show essential enzymes catalysing these reactions and requiring ThPP as a co-factor (shown in green). Green oval indicates ThPP. Red boxes indicate the positions inside a cell. The image is modified from (Hohmann and Meacock, 1998).

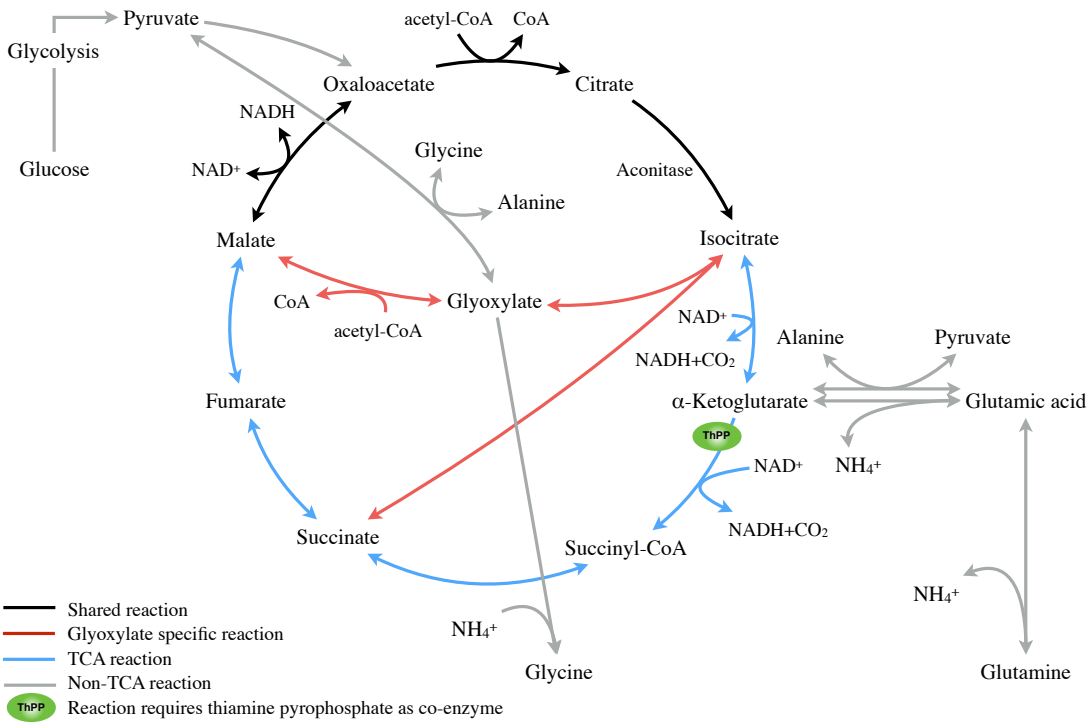


Figure 2-11: Simplified schematic of TCA and glyoxylate cycles from the central metabolism in eukaryotic cells. Black lines indicate reactions shared in both glyoxylate cycle and TCA cycle. Red and blue lines indicate reactions specific to the glyoxylate and TCA cycle, respectively. Grey lines indicate reactions not presented in either cycle. The image is modified from (Lorenz and Fink, 2002).

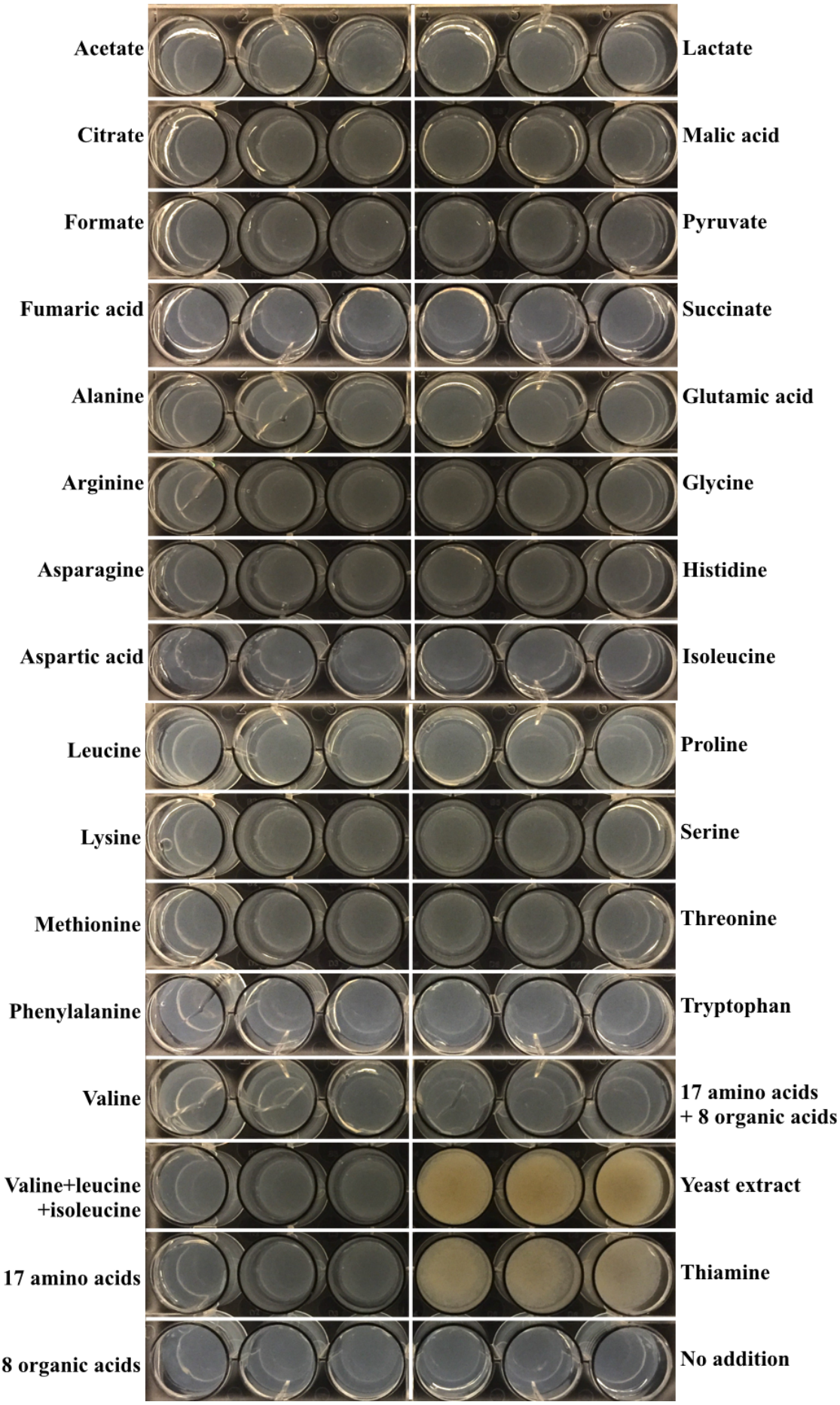

Acetate
Lactate
Citrate
Malic acid
Formate
Pyruvate
Fumaric acid
Succinate
Alanine
Glutamic acid
Arginine
Glycine
Asparagine
Histidine
Aspartic acid
Isoleucine
Leucine
Proline
Lysine
Serine
Methionine
Threonine
Phenylalanine
Tryptophan
Valine
17 amino acids
+ 8 organic acids
Valine+leucine
+isoleucine
Yeast extract
17 amino acids
Thiamine
8 organic acids
No addition

Figure 2-12: Amino acid and organic acid screen on *S. indica* growth. Two weeks' *S. indica* growth on the media containing different amino acid or organic acid as supplement. Each treatment has three replicates presented in 3 adjacent wells. *S. indica* growth is visible as white-yellow colonies on the surface of a well. Treatments with yeast extract and thiamine served as positive controls. Treatments without addition (only the basic medium) served as negative controls. The diameter of each well is 16.5 mm. Images were taken after two weeks of growth. Two biological replicates of this experiment were performed with qualitatively similar results.

2.4.4 *S. indica* thiamine auxotrophy is reflected in its genomic enzyme content

To support and better understand these physiological results, the *S. indica* genome has been analysed for the presence of genes associated with thiamine utilisation, biosynthesis, and transportation (Figure 2-13) (Table 2-3).

Table 2-3: Results of *S. indica* BLAST analysis, in which *S. indica* genome is searched against thiamine related genes from *S. cerevisiae*.

Gene name	Functions	Source organism for sequence	Accession of homologous in *S. indica*
THI6	1.hydroxyethylthiazole kinase 2.thiamine-phosphate pyrophosphorylase (Hohmann and Meacock, 1998)	*Saccharomyces cerevisiae* S288c	No
THI20	1.HMP kinase 2.HMP-P kinase 3.Thiaminase II (Haas *et al.*, 2005)		No
THI4	1.Thiamine thiazole synthase 2.Mitochondrial DNA damage tolerance (Machado *et al.*, 1997)		CCA73069.1
THI5	Pyrimidine precursor biosynthesis (Wightman, 2003)		No
THI80	Thiamine pyrophosphokinase (Fankhauser *et al.*, 1995)		CCA69955.1
PHO3	Thiamin-repressible acid phosphatase (Wightman, 2003)		CCA74294.1
THI7 (THI10)	Thiamin transporter (Hohmann and Meacock, 1998)		CCA72717.1

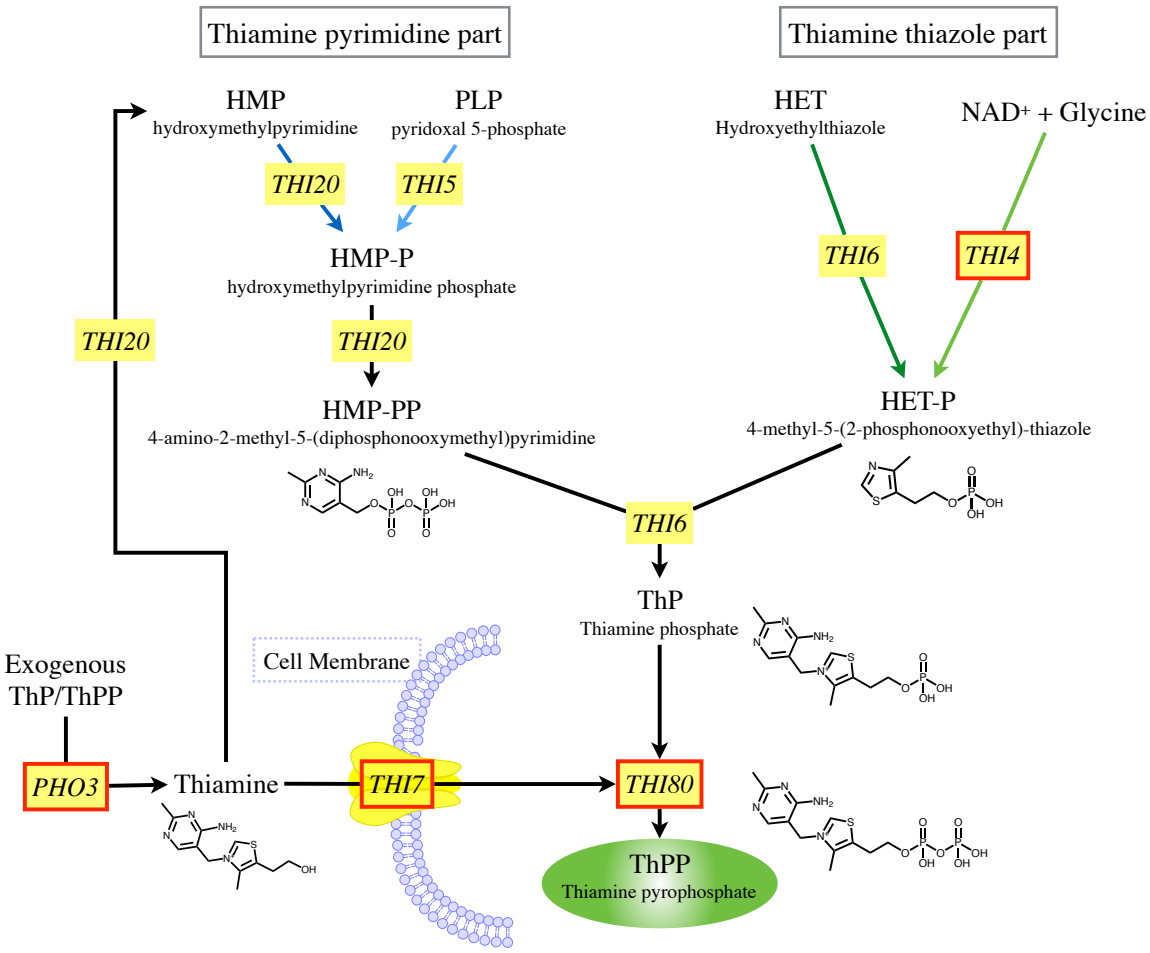


Figure 2-13: Overview of the thiamine biosynthesis and salvage pathway in *Saccharomyces cerevisiae* based on (Wightman, 2003), and as included in the KEGG metabolic pathways (Pathway: sce00730) (Kanehisa and Goto, 2000). Yellow boxes indicate genes encoding for the enzymes in the corresponding reactions. Red borders indicate genes for which there are *S. cerevisiae* homologues in *S. indica* (see Methods). Light and dark blue or light and dark green arrows indicate parallel pathways are generating the same thiamine precursor respectively. The image is modified from (Hohmann and Meacock, 1998).

This bioinformatics analysis revealed that *S. indica* lacks most of the genes of the thiamine biosynthesis pathway (Figure 2-13) (Table 2-3). In particular, no homologs of the genes *THI5, THI6*, and *THI20* was found. *THI5* encodes the enzyme involved in the synthesis of the thiamine-precursor hydroxymethylpyrimidine (HMP) (Wightman, 2003), *THI6* encodes the bifunctional enzyme acting as thiamine

phosphate pyrophosphorylase and hydroxyethylthiazole kinase (Hohmann and Meacock, 1998), and *THI20* encodes a HMP kinase that displays both kinase and thiaminase II activity (Haas *et al.*, 2005). For the gene *THI4*, which encodes a bifunctional protein involved in thiamine thiazole synthase and mitochondrial DNA damage tolerance (Machado *et al.*, 1997; Hohmann and Meacock, 1998; Wightman, 2003), a truncated homolog (11% length coverage against the *THI4* sequence in *S. cerevisiae*) was found in *S. indica*. Considering all other key biosynthesis genes (*THI5*, *THI6*, and *THI20*) are absent, it is possible that this short form relates to mitochondrial DNA damage tolerance (Machado *et al.*, 1997; Hohmann and Meacock, 1998; Wightman, 2003) rather than thiamine biosynthesis. *S. indica* was also found to contain a homolog of the *THI7* (alternative name *THI10*) that encodes a thiamine transporter (Hohmann and Meacock, 1998), and a homolog of the *PHO3* gene, whose product catalyzes the dephosphorylation of thiamine phosphate to thiamine, thereby increasing its uptake (Hohmann and Meacock, 1998). The *S. indica* genome also contains *THI80*, which encodes a thiamine pyrophosphokinase catalyzing the conversion of thiamine into thiamine pyrophosphate (ThPP) (Fankhauser *et al.*, 1995).

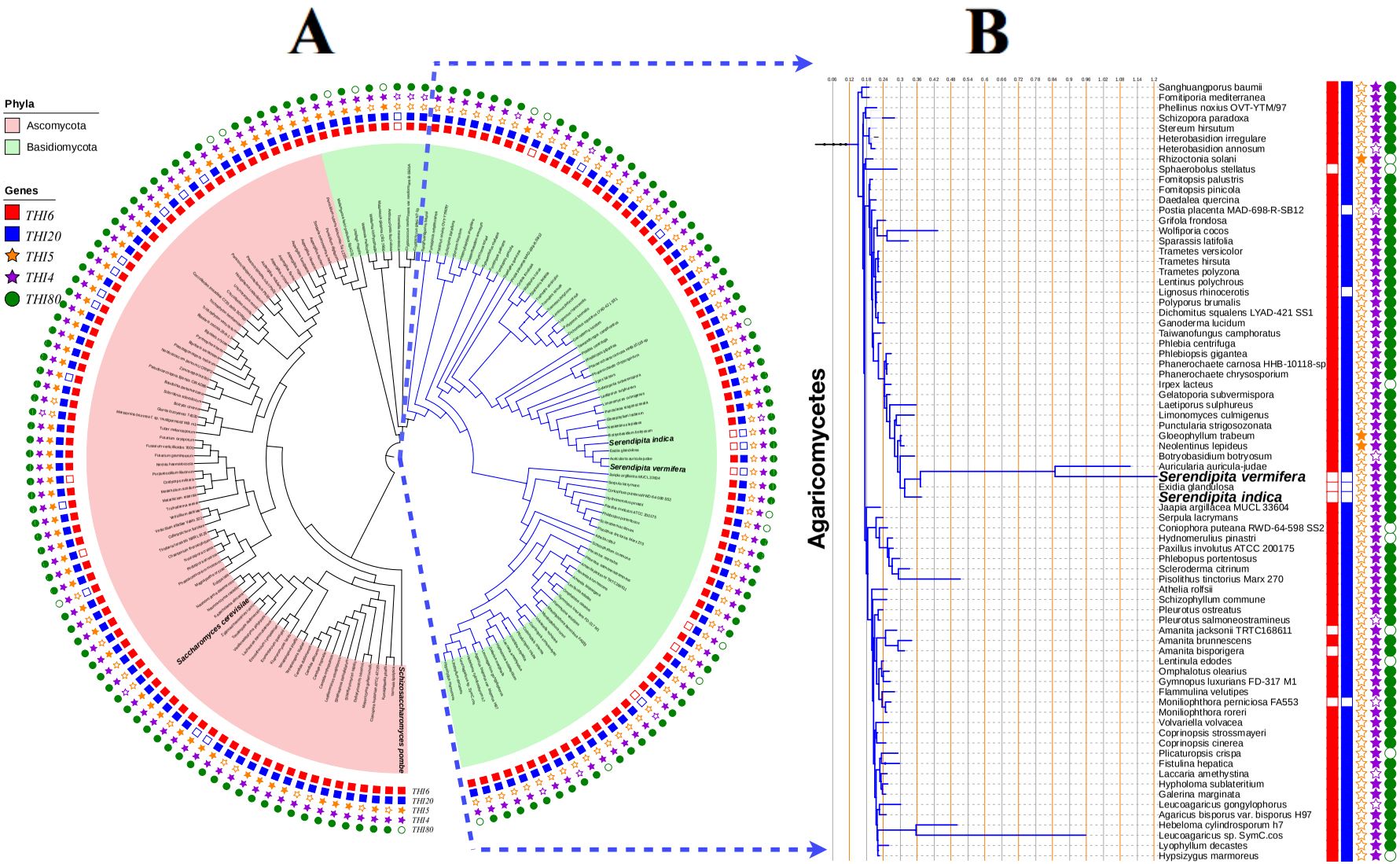
2.4.5 Thiamine auxotrophy is not only in *S. indica*

The above results (section 2.4.1 to 2.4.4) support the experimental observation of thiamine auxotrophy in *S. indica*. To see if such a loss of thiamine biosynthesis is wide-spread in other fungi, including evolutionarily closely and more distant relatives of *S. indica* and unrelated fungal species, an analysis has been done on the absence and presence of all of the key genes (*THI4*, *THI5*, *THI6*, *THI20*, *THI7*, *PHO3* and *THI80*) in available 162 fungal genomes in the phyla of Basidiomycota (featuring *S. cerevisiae*) and Ascomycota (featuring *S. indica*) (Appendix A). This information was

mapped onto a phylogenetic tree created using the 18s ribosomal gene. Summarized in Figure 2-14, this analysis showed the same genetic pattern (including the truncated version of the *THI4* homolog) for key thiamine biosynthesis genes in *Serendipita vermifera*, the closest relative (with full genome assembly information) of *S. indica*. In addition, within the analysed species from the Basidiomycota phylum, there are 5 species lacking either *THI20* or *THI6*, and 5 species lacking both genes, suggesting that they are unable to synthesise thiamine on their own (Figure 2-13). Among the analysed species from the Ascomycota phylum, there are 14 species lacking either *THI20* or *THI6*, while none of them lacks both genes. Taken together, these findings suggest that a complete or partial loss of thiamine biosynthesis genes can be observed in different fungi from different classes, rather than being confined to a specific class.

(Figure is on next page)

Figure 2-14: Phylogenetic tree of 162 fungal species with the indication of essential thiamine biosynthesis genes. **(A).** The phylogenetic tree of all 162 fungi species used. Key species, *S. cerevisiae* (budding yeast), *Schizosaccharomyces pombe* (fission yeast), *S. indica* and *S. vermifera* are highlighted in bold. The tree contains 77 species from the phylum of Ascomycota (red background) and 85 species from the phylum of Basidiomycota (green background). Of the latter, 76 species are in the class of Agaricomycetes (blue branches). **(B).** Closer view of the phylogenetic relations among all 76 species in the class of Agaricomycetes. On both **(A)** and **(B)**, the presence/absence of each of the five thiamine biosynthesis genes (*THI6*, *THI20*, *THI5*, *THI4*, *THI80*) in each species are indicated with different colours and symbols (as shown on the legend). Filled (empty) symbols indicate the presence (absence) of a gene in each species.


A
B
Phyla
Ascomycota
Basidiomycota
Genes
THI6
THI20
THI5
THI4
THI80
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Serendipita indica
Serendipita vermifera
Agaricomycetes
Sanghuangporus baumii
Fomitiporia mediterranea
Phellinus noxius OVT-YTM/97
Schizopora paradoxa
Stereum hirsutum
Heterobasidion irregulare
Heterobasidion annosum
Rhizoctonia solani
Sphaerobolus stellatus
Fomitopsis palustris
Fomitopsis pinicola
Daedalea quercina
Postia placenta MAD-698-R-SB12
Grifola frondosa
Wolfiporia cocos
Sparassis latifolia
Trametes versicolor
Trametes hirsuta
Trametes polyzona
Lentinus polychrous
Lignosus rhinocerotis
Polyporus brumalis
Dichomitus squalens LYAD-421 SS1
Ganoderma lucidum
Taiwanofungus camphoratus
Phlebia centrifuga
Phlebiopsis gigantea
Phanerochaete carnosa HHB-10118-sp
Phanerochaete chrysosporium
Irpex lacteus
Gelatoporia subvermispora
Laetiporus sulphureus
Limonomyces culmigenus
Punctularia strigosozonata
Gloeophyllum trabeum
Neolentinus lepideus
Botryobasidium botryosum
Auricularia auricula-judae
Serendipita vermifera
Exidia glandulosa
Serendipita indica
Jaapia argillacea MUCL 33604
Serpula lacrymans
Coniophora puteana RWD-64-598 SS2
Hydnomerulius pinastri
Paxillus involutus ATCC 200175
Phlebopus portentosus
Scleroderma citrinum
Pisolithus tinctorius Marx 270
Athelia rolfsii
Schizophyllum commune
Pleurotus ostreatus
Pleurotus salmoneostramineus
Amanita jacksonii TRTC168611
Amanita brunnescens
Amanita bisporigera
Lentinula edodes
Omphalotus olearius
Gymnopus luxurians FD-317 M1
Flammulina velutipes
Moniliophthora perniciosa FA553
Moniliophthora roreri
Volvariella volvacea
Coprinopsis strossmayeri
Coprinopsis cinerea
Plicaturopsis crispa
Fistulina hepatica
Laccaria amethystina
Hypholoma sublateritium
Galerina marginata
Leucoagaricus gongylophorus
Agaricus bisporus var. bisporus H97
Hebeloma cylindrosporum h7
Leucoagaricus sp. SymC.cos
Lyophyllum decastes
Hypsizygus marmoreus

2.5 Discussion

The growth condition screen experiments presented in this chapter suggested the thiamine auxotrophy of *S. indica*. The performed bioinformatics analysis on *S. indica* confirmed this discovery. This is the first report on *S. indica* inability to synthesis thiamine, which would be a crucial factor for managing *S. indica* cultivation, and analysing *S. indica* interaction with other organisms. For example, in the mass cultivation of *S. indica* as biofertilizer (Singhal *et al.*, 2017a), media used for optimal *S. indica* cultivation all contained thiamine or other ingredients providing thiamine (such as yeast extract), but the thiamine necessity was not pointed out. Also, in the metabolome analysis of plant *Arabidopsis thaliana* when it being colonized by *S. indica* (Strehmel *et al.*, 2016), authors found thiamine abundance in root extracts changed after *S. indica* colonization, and the addition of vitamin mixture (containing thiamine) to be essential in establishing the symbiosis between *S. indica* and *A. thaliana*. However, thiamine was one of many metabolites analysed in that study, and no importance of thiamine was further discussed.

The developed synthetic medium with defined elements for *S. indica* is important in ruling out unknown factors that might affect the analysis on *S. indica* growth. For example, in the experiment testing different nitrogen sources and thiamine concentrations on *S. indica* growth reported in this study, by knowing the thiamine auxotrophy of *S. indica*, the conclusion could be drawn that ammonium and glutamine can support *S. indica* growth while nitrate cannot. This is consistent with previous research (Zuccaro *et al.*, 2011), in which nitrogen preference of *S. indica* was tested on a commercial medium with a clear list ingredients on macro- and micro-elements, salts and vitamins used (Yeast nitrogen base agar medium without amino acids and ammonium sulphate (DIFCO, REF 233520) with 20% glucose was

used as carbon source). However, in another study researchers trying to identify favourable nitrogen source of *S. indica* (Kumar *et al.*, 2011), the conclusion was made that yeast extract and peptone help in *S. indica* biomass accumulation, while the effect of ammonium supporting *S. indica* growth was not detected. Although similar ammonium level (28mM) was used as in the synthetic medium in this study (20mM, Table 2-1), also similar pH and buffer strength, their conclusion was not in accordance to this study. This could be explained by the fact that when they were using ammonium as a nitrogen source, no elements in the medium was providing thiamine (yeast extract and peptone as thiamine source in Kaefer medium (Pham *et al.*, 2008) was removed).

The phylogenetic analysis shows that these genetic patterns are not unique to these two species, but also not widespread, indicating that loss of thiamine biosynthesis can be related to their ecology and lifestyle (both species are general plant colonisers (Weiss *et al.*, 2016)). Within an environment where thiamine is readily available, it is indeed possible that thiamine biosynthesis is lost due to enzymatic costs. In particular, the protein product of *THI4* is shown to undergo only a single turnover before requiring re-synthesis, a so-called "suicide enzyme" (Chatterjee *et al.*, 2011). Due to this potential high cost, loss of *THI4* related thiamine biosynthesis might be adaptive under circumstances where thiamine can be acquired from the environment (Kraft and Angert, 2017). In both *S. indica* and its close relative *S. vermifera* genome there is only a truncated *THI4* gene, and it is possible that this gene relates only to the known additional function in mitochondrial DNA repair (Machado *et al.*, 1997; Chatterjee *et al.*, 2011) rather than thiamine biosynthesis (as the other thiamine biosynthesis gene *THI6*, *THI20* and *THI5* are absent). In addition, within the analysed species from the Basidiomycota phylum, there are 5 species

lacking either *THI20* or *THI6*, and 5 species lacking both genes, suggesting that they are unable to synthesise thiamine on their own. Among the analysed species from the *Ascomycota* phylum, there are 14 species lacking either *THI20* or *THI6*, while none of them lack both genes. Taken together, these findings suggest that a complete or partial loss of thiamine biosynthesis genes can be observed in different fungi from different classes, rather than being confined to a specific class. This might relate to the ecology and lifestyle of these species, which would require further analyses in future.

S. indica bears the inability to synthesis thiamine, while thiamine is a necessary need for its central metabolism. On the other hand, thiamine can be produced by many plants, fungi and bacteria via various mechanisms (Jurgenson *et al.*, 2009; Helliwell *et al.*, 2013). According to the Black Queen Hypothesis, access to a public good could lead to the loss of essential genes of synthesising it in an individual from that microbial community (Morris *et al.*, 2012). If *S. indica* lost such pathway to reduce the cost of living, its survival would have to rely on the interactions with other organisms heavily. Therefore, thiamine could serve as a key factor linking *S. indica* with other organisms, and the synthetic medium developed in this chapter would be a controlled environment for constructing a model system to study microbial interactions. The construction of such system is discussed in the following chapter.

Chapter 3

Synthetic microbial community of

Serendipita indica and *Bacillus subtilis*

Most parts of this chapter are included in the author's publication Jiang, X. *et al.*, 2018, except the section 3.1, part of section 3.3 and 3.5 and the figure 3-11.

3.1 Abstract

Towards understanding and managing the plant-beneficial microbial community in the rhizosphere, and overcoming the difficulty of analysing complex natural community, a reductionist approach was applied to build a synthetic system using ecologically and functionally relevant species: the plant beneficial endophytic fungus *Serendipita indica,* and the soil-dwelling model bacterium *Bacillus subtilis*. *S. indica* growth was found to be restored in thiamine-free media, when co-cultured with *B. subtilis*. Cross-feeding between those two species was found using metabolic profiling methods. The success of this auxotrophic interaction was dependent on the spatial and temporal organization of the system; the beneficial impact of *B. subtilis* was only visible when its inoculation was separated from that of *S. indica* either in time or space.

3.2 Introduction

Higher-level functions and population dynamics within microbial communities are underpinned by the interactions among the composing species within the community and their environment (Falkowski *et al.*, 2008; Sañudo-Wilhelmy *et al.*, 2014). Deciphering these interactions is a prerequisite to understand and manage complex natural communities (Abreu and Taga, 2016) and to achieve community-level synthetic engineering (Großkopf and Soyer, 2014; Hays *et al.*, 2015; Lindemann *et al.*, 2016). To this end, increasing numbers of experimental studies and (meta)genomic surveys have shown that auxotrophic interactions, involving vitamins

and amino acids, are widespread in many natural microbial communities (Morris *et al.*, 2012; Helliwell *et al.*, 2013; Sañudo-Wilhelmy *et al.*, 2014; Campbell *et al.*, 2015; Romine *et al.*, 2017) and can also be engineered genetically to create synthetic communities (Campbell *et al.*, 2016; Mee *et al.*, 2014; Pande *et al.*, 2014). Specific auxotrophic interactions among microbes are shown to influence ecosystem functioning; for example, infection outcomes within higher organisms (Wargo and Hogan, 2006), ecological population dynamics in the oceans (Sañudo-Wilhelmy *et al.*, 2014), and the levels of biodegradation of organic matter under anoxic conditions (Schink, 1997; Embree *et al.*, 2015).

It has been suggested that auxotrophies can result from reduced selective pressures for maintaining biosynthesis capabilities under stable metabolite availability due to abiotic or biotic sources (Morris *et al.*, 2012; Helliwell *et al.*, 2013). This proposition is supported by the observed independent evolution of vitamin and amino acid auxotrophies in different, unrelated taxa (Helliwell *et al.*, 2011; Rodionova *et al.*, 2015), and points to a direct linkage between ecological dynamics and evolution of auxotrophies (Embree *et al.*, 2015). The possible fluctuations in metabolite availabilities in time and space would be expected to impact both the emergence of auxotrophies and the population dynamics of resulting auxotrophic species. For example, in the marine environment, where the observed auxotrophies relate mostly to the loss of biosynthesis capacity for vitamins and amino acids, population dynamics of auxotrophic species are believed to be directly linked to those of "provider" species (Helliwell *et al.*, 2013; Hom and Murray, 2014; Sañudo-Wilhelmy *et al.*, 2014). The ecological influences of auxotrophic species on community structure and population dynamics can also be exerted by abiotic fluctuations or directly by the abundances and actions of higher organisms within the system.

These ecological influences on microbial population dynamics can increase significantly in spatially organized systems. Nevertheless, the spatial context of microbial interactions is only beginning to be appreciated (Ratzke and Gore, 2016; Momeni *et al.*, 2013; Müller *et al.*, 2014; Campbell *et al.*, 2015). Considering that each species can display multiple metabolic actions and that all of these can affect a shared environment, it is not clear if auxotrophic interactions are always successfully established even if genetic or metabolic complementarity is present. For example, metabolic interactions among genetically engineered auxotrophic yeast are shown to require an initial autonomous growth period to establish (Campbell *et al.*, 2015). Establishment of these metabolic interactions might also involve spatial factors including the formation of metabolic gradients and specific population organizations (Müller *et al.*, 2014; Momeni *et al.*, 2013; Carmona-Fontaine *et al.*, 2017; Embree *et al.*, 2015). These spatial factors can include oxygen and pH gradients, as both of these can significantly change upon the growth of one species (Vylkova, 2017; Peters and Wimpenny, 1987; Dietrich *et al.*, 2013), and can directly influence subsequent or simultaneous metabolic interactions or growth of different species. Studies exploring the possible interplays between species-species and species-environment interactions have so far used synthetically engineered interactions (Campbell *et al.*, 2015; Momeni *et al.*, 2013; Müller *et al.*, 2014) or enriched microbial communities (Embree *et al.*, 2015). Further analyses of such interplay in ecologically and biotechnologically relevant systems can thus help engineering of novel applications of microbial communities with inherent spatial organization, such as those seen in agriculture and involving, for example, closed-ecosystem production, seed treatment, and microbe-based biofertilization (Gòdia *et al.*, 2002; Lucy *et al.*, 2004; Richardson *et al.*, 2011).

Towards this goal, the focus of this chapter is to identify potential metabolic interactions between the plant beneficial endophytic fungus *Serendipita indica* and the ubiquitous soil microbe *Bacillus subtilis*. *B. subtilis* is one of the model organism for studying thiamine biosynthesis, contains a complete thiamine biosynthesis pathway (Begley *et al.*, 1999). It also forms beneficial interactions with plants when colonising the roots (Allard-Massicotte *et al.*, 2016). In the synthetic co-culture, *S. indica* thiamine auxotrophy could be satisfied and its growth restored by *B. subtilis*. The success of this auxotrophic interaction, however, was strongly dependent on the temporal and spatial organization in the system.

These findings and the established synthetic co-culture could act as a basis to develop a more complete functional synthetic community, as advocated for biotechnological applications and for gaining insights into community function (Großkopf and Soyer, 2014; Lindemann *et al.*, 2016; Mee and Wang, 2012; Widder *et al.*, 2016).

3.3 Materials and methods

Analytical-grade chemicals were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA), or Fisher Scientific UK (Loughborough, UK).

3.3.1 *S. indica* spore suspension preparation

S. indica spore suspensions were prepared using the method mentioned in section 2.3.2. The final concentration of spore suspension was always 500,000 spores per ml. *S. indica* spore suspensions were prepared freshly on the same day or the day before inoculation for each experiment.

3.3.2 *B. subtilis* culture preparation

Cryostock of *B. subtilis* (strain NCIB3610) was re-inoculated in 5ml LB medium (Bertani, 1951) and incubated overnight at 37 ºC, 200 rpm. The overnight culture was sub-cultured 10 µl to 5ml fresh LB medium and incubated for another 4 to 6 hours, until OD_{600} reached 0.5 determined by spectrophotometer (Spectronic 200, Thermo Fisher Scientific). This culture was then centrifuged at 3000 g for 2 min for spinning down *B. subtilis* cells. The supernatant was discarded and replaced with sterile 10mM $MgCl_2$. This step was repeated twice for washing off unwanted metabolites from the old culture, leaving only *B. subtilis* cells in the 10 mM $MgCl_2$ solution to balance cellular osmotic pressure (Qi and Adler, 1989). The washed *B. subtilis* culture was re-suspended and adjusted to OD_{600} 0.5 with 10 mM $MgCl_2$.

3.3.3 *S. indica* interaction with *B. subtilis* on agar synthetic medium

The co-culturing of *S. indica* and *B. subtilis* was initiated on agar medium as indicated in Figure 3-1. Experiments were carried out on 60 mm petri dishes (Ref:

1007, Corning), filled with 6 ml of agar medium prepared as mentioned in section 2.3.1. Synthetic media without thiamine and containing ammonium or glutamine as the sole nitrogen source were used to create different environments for the microbial interaction. *S. indica* spore suspension was inoculated 2 µl on the left side of the plates. At approximately 2 cm distance to the right of the inoculum, either a 2 µl "mock" solution (10mM $MgCl_2$) or a 2 µl *B. subtilis* culture were placed. Plates were sealed with parafilm and incubated statically at 30℃ for 2 weeks. Images were taken with a gel doc system (G:Box EF, Syngene).

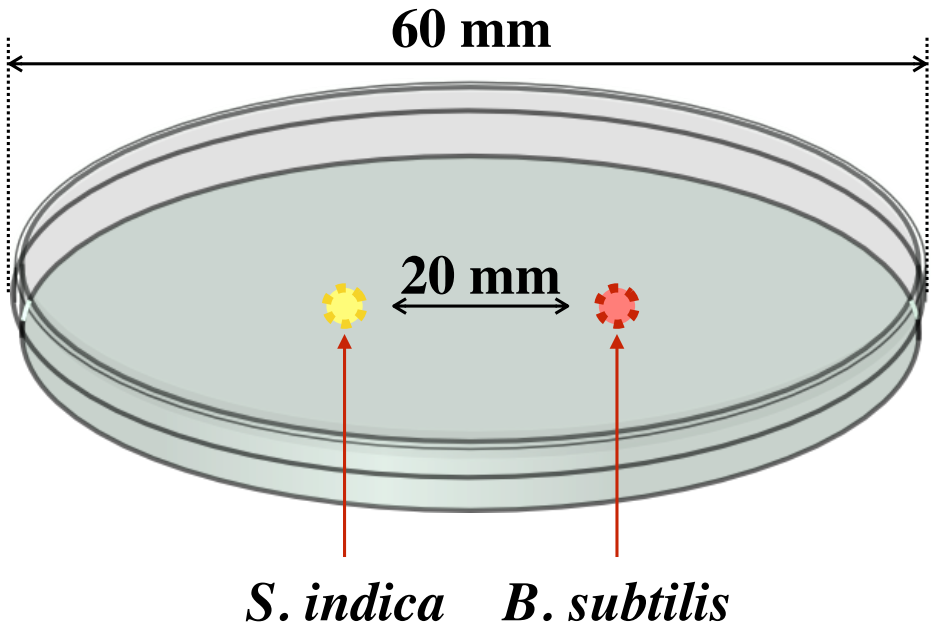


Figure 3-1: Inoculation scheme of *S. indica* and *B. subtilis* inoculation on agar plate. *S. indica* inoculum is shown in the yellow circle and *B. subtilis* inoculum is in the red circle. The diameter of the plate is 60 mm. A space of 20 mm is in between *S. indica* and *B. subtilis*.

3.3.4 Supernatant cross-feeding experiments and metabolites analysis

Axenic cultures of *S. indica* and *B. subtilis* were cultivated in 50 ml synthetic medium described in section 2.3.1, with different nitrogen sources (ammonium or glutamine) and different thiamine conditions (with/without). For *S. indica* an

inoculum of 50 µl spore suspension prepared as described in section 2.3.2 was used. For *B. subtilis*, an inoculum of 50 µl OD_{600} 0.5 culture prepared as described in section 3.3.2 was used. Cultures were shaking incubated for 1 week at 30 °C and 150 rpm. After one-week growth, *S. indica* cells were harvested by centrifugation at 18,000 g for 20 min. The supernatant was collected and filtered through a 0.2 µm polyethersulfone (PES) filter (Ref: WHA67802502, Whatman), while biomass was washed with 40 ml MilliQ water, dried using a centrifugal evaporator (EZ-2 Elite, Genevac), and then weighed. The one-week growth of *B. subtilis* in liquid cultures was monitored by taking 1 ml samples and measuring OD_{600} by spectrophotometer (Spectronic 200, Thermo Fisher Scientific). The remaining liquid culture was centrifuged at 18,000 g for 10 min. The supernatant was collected and filtered through a 0.2 µm PES filter, while biomass in the pellet was discarded.

Supernatants from *S. indica* or *B. subtilis* were mixed with fresh medium (same recipe as each initial culture respectively) in a 1:1 ratio to set up new axenic cultures of *S. indica* and *B. subtilis*. The mixed fresh medium served as nutrition guarantee in case the nutrients in the supernatant was consumed entirely. Same inoculum scheme was used for *S. indica* and *B. subtilis*. *S. indica* was inoculated in *B. subtilis* supernatant (mixed with fresh medium); vis versa *B. subtilis* was inoculated in *S. indica* supernatant (mixed with fresh medium). The same procedure was performed for the cultivation and sample collection and growth recording (Figure 3-2).

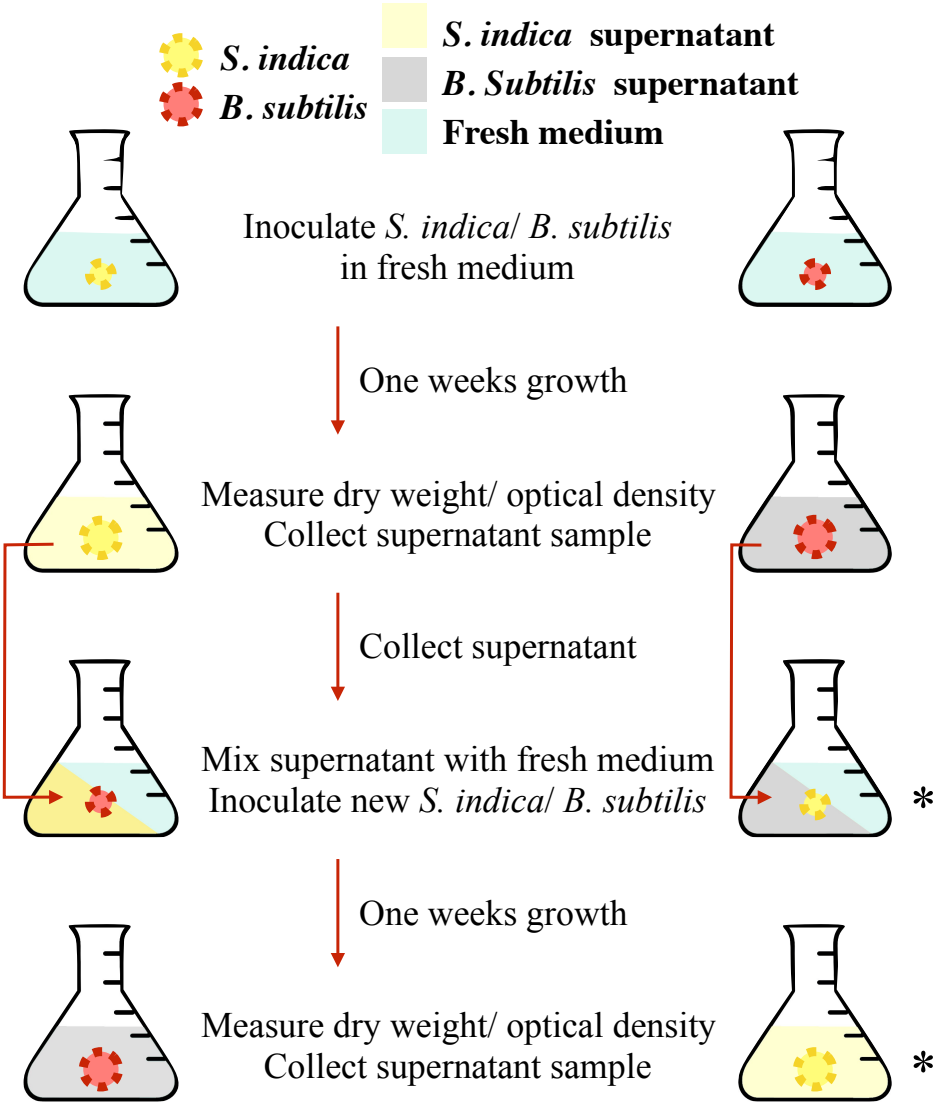


Figure 3-2: Cultivation scheme of the cross-feeding experiment. Detailed cultivation procedure is described in section 3.3.4. *S. indica* and *B. subtilis* are indicated with a yellow and red circle respectively. Fresh medium and *S. indica* and *B. subtilis* are indicated with cyan, yellow and grey. Supernatant of each drawn flasks were used for IC analysis. Supernatants of flasks indicated with * were used for amino acid analysis.

Liquid samples were collected 1 ml from these cultures at each stage by filtering through a 0.2 µm nylon membrane (Ref: WHA7402004, Whatman) to remove microbes. Samples collected were indicated on Figure 3-2: initiating the culture of one organism, after one-week growth of this organism, mixing supernatant

with fresh medium, after one-week growth of the other organism. Samples were transferred into polypropylene vials (Ref: 079812, Thermo Fisher Scientific) for Ion Chromatography (IC), which was performed using Dionex ICS-500^{+} and column Dionex IonPac AS11-HC-4 μm (2 × 250 mm). Standard curves of each metabolite in IC were prepared by dissolving known chemicals at specific concentrations (at least 5 concentrations in the range of expected concentration in samples) in both MilliQ water and synthetic medium and running through IC system using suggested elute method from column manufacture manual. The retention times of each metabolite were recorded using IC operating software Chromeleon and were compared with the run results of each sample for the detection of each metabolite.

Samples from the same preparation method were sent for a commercial amino acid analysis (performed by Genaxxon, Ulm, Germany). A polymeric cation exchanger was used to separate amino acids by high-performance liquid chromatography (particle size: 5 μm; column dimensions: 125 × 4.6 mm ID) (Amino Acid Analyzer LC3000). Separated amino acids were detected by post-column Ninhydrin derivatization at 125 °C and photometric measurement at 570 nm. Due to budget constraints, only samples from the thiamine-free medium, before inoculation and after one-week growth of *S. indica* in the mixture of *B. subtilis* supernatant and fresh medium were analysed in this method (the two bottom right samples indicated with * in Figure 3-2).

3.3.5 Thiamine measurements on *B. subtilis* liquid cultures

Axenic cultures of *B. subtilis* were cultivated in three replicates of 50 ml synthetic medium containing glutamine as a sole nitrogen source and without thiamine. *Bacillus subtilis* inoculum of 50 μl prepared as described in section 3.3.2

was used. After 1 week of shaking incubation in 30 °C and at 150 rpm, *B. subtilis* cultures were harvested by centrifugation at 18,000 g for 10 min. The supernatant was collected and filtered through a 0.2 nm PES membrane filter (Ref: WHA67802502, Whatman). The supernatants of 250 µl from each culture were transferred to a clean 1.5 ml Eppendorf tube, followed by sequentially adding 10 µl 1% (w/v) $K_3[Fe(CN)_6]$, 150 µl 15% (w/v) NaOH solution, and 150 µl isobutanol. The tubes were shaken vigorously for 1 min, followed by 2 min of centrifugation at 13,000 g. The upper isobutanol layer of each tube was transferred to a new 1.5 ml Eppendorf tube, containing 0.2 g Na_2SO_4. The tubes were mixed thoroughly and centrifuged for 1 min at 13,000 g for solids to settle. One hundred microliters of supernatant from each tube were transferred to 96-well plates (Ref: 3916, black flat bottom, Corning) and the fluorescence was measured using a plate reader (CLARIOstar, BMG Labtech) at 365 nm excitation and 450 emission. The concentration of thiamine was determined with a series of known concentration standard thiamine solutions under the same treatment.

3.3.6 Spatial and temporal separation experiments

S. indica (spore suspension prepared as described in section 2.3.2) and *B. subtilis* (culture prepared as described in section 3.3.2) were cultivated on thiamine-free synthetic medium containing ammonium as the sole nitrogen source and six-well tissue culture plates (Ref: 353046, Falcon) were used. On each plate, 1 µl of *S. indica* and 1 µl of *B. subtilis* were inoculated on 5 of the wells; one well was intentionally left non-inoculated as a blank for later image analysis (Figure 3-3). In the "no separation" case, *S. indica* and *B. subtilis* were pre-mixed at 1:1 volume ratio and inoculated on the centre of each well. In the "spatial separation" case, *S. indica* was

inoculated 7.5 mm left to the centre of a well and *B. subtilis* 7.5 mm right to the centre, leaving 15 mm distance in between. In the "temporal separation" case, *S. indica* was inoculated on the centre of each well, and the plates were then incubated for 3 days and *B. subtilis* was inoculated on the centre of each well after this time. The 3-day time separation was primarily based on the germination time of *S. indica* spores, which can take 2–3 days in the synthetic medium. In the "Spatial-temporal separation" case, *S. indica* was inoculated 7.5 mm left to the centre of a well, and the plates were then incubated for 3 days and *B. subtilis* was inoculated 7.5 mm right to the centre of a well after this time. All the plates were incubated in 30 °C for 2 weeks (counting starts from the time of *S. indica* inoculation). Images were taken by scanning each well under a microscope (Olympus IX83) under bright field. ImageJ was used for measuring the biomass by integration of the total colony density. An image of each colony was manually outlined using the selection tool. The selected area was compared with the same location on the blank well from the same plate. The area and relative intensity were recorded (using "measure" function) and used for calculating the colony growth.

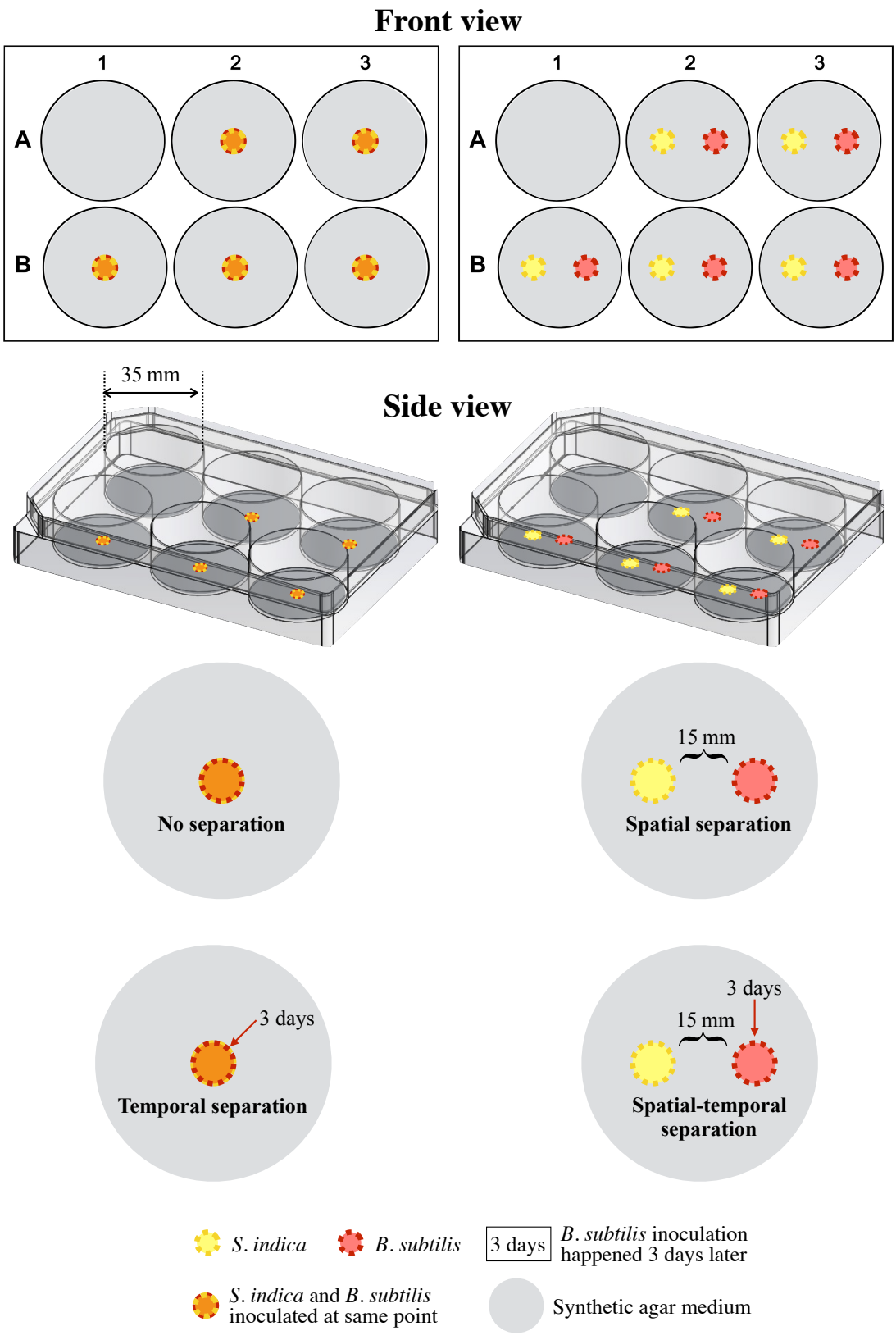


Figure 3-3: Inoculation scheme of *S. indica* and *B. subtilis* on 6-well plate for spatial and temporal separation experiment. The A1 position on the plate contains medium is intentionally left un-inoculated for serving as a blank for image analysis. The diameter of each well is 35 mm.

3.3.7 Oxygen measurements

Oxygen measurements were done in a replicate experiment with the "spatial separation" and "no separation" treatments described in section 3.3.6 using the thiamine-free agar medium containing glutamine as the sole nitrogen source. UniSense microelectrode (OX-NP with Unisense microsensor multimeter and Unisense Sensor Trace Suite software) was used for daily oxygen measurements. Three identical plates of the same treatment and initiated on the same day was used for each measurement (i.e., three plates per day per treatment) and discarded afterwards. On each day, the micro-electrode was calibrated using aerated distilled water (air bubbled through for 3–5 min) and an anaerobic solution (prepared as per the manufacturer's instructions; 0.1 M ascorbic acid in 0.2 M NaOH, mixed briefly to dissolve and left unagitated for calibration 5 min prior to the first measurement). For each measurement, the electrode was first placed on the colony or agar surface. Once a measurement at the surface was done, the electrode was lowered into the agar or colony with the help of a manual micro-manipulator (Scientifica), and at 400 µm intervals in the z-dimension of the manipulator. Three depths (0.4, 0.8, and 1.2 mm from the surface) were used for oxygen measurement. Measurements were taken on *B. subtilis*, *S. indica*, and pre-mixed co-culture inoculation points, as well as on an off-location without any bacteria or fungi growth.

3.4 Results

3.4.1 *B. subtilis* complements *S. indica's* auxotrophy for thiamine and promotes its growth

Given the crucial role of thiamine-derived co-factors in central metabolism (Figure 2-10), *S. indica* growth in nature apparently depends on environment-derived thiamine which can be synthesised by various bacteria, fungi, and plants (Begley *et al.*, 1999; Jenkins *et al.*, 2007; Jurgenson *et al.*, 2009). Among these, *B. subtilis*, a bacterium commonly found in the soil (Hong *et al.*, 2009) is an established model organism (Mäder *et al.*, 2012). *Bacillus subtilis* also requires thiamine for its growth and its thiamine biosynthesis pathways are well studied (Begley *et al.*, 1999; Schyns *et al.*, 2005). Combined with the fact that *B. subtilis* is commonly a plant-beneficial microbe (Castillo *et al.*, 2013), this motivated me to explore the possibility that the identified *S. indica* auxotrophy for thiamine could be satisfied upon co-culturing with *B. subtilis*. Co-cultures of these two species were created on agar plates using the defined thiamine-free synthetic medium, and two common nitrogen sources (ammonium and glutamine) to evaluate possible auxotrophic interactions under these conditions. *B. subtilis* could indeed stimulate *S. indica* growth under thiamine-free conditions and that *S. indica* growth followed a spatial pattern, with significant growth in the vicinity of the *B. subtilis* colony (Figure 3-4). A similar spatial growth was observed in experiments with *S. indica* and a supplied thiamine agar block (Figure 2-7) described in section 2.4.1.

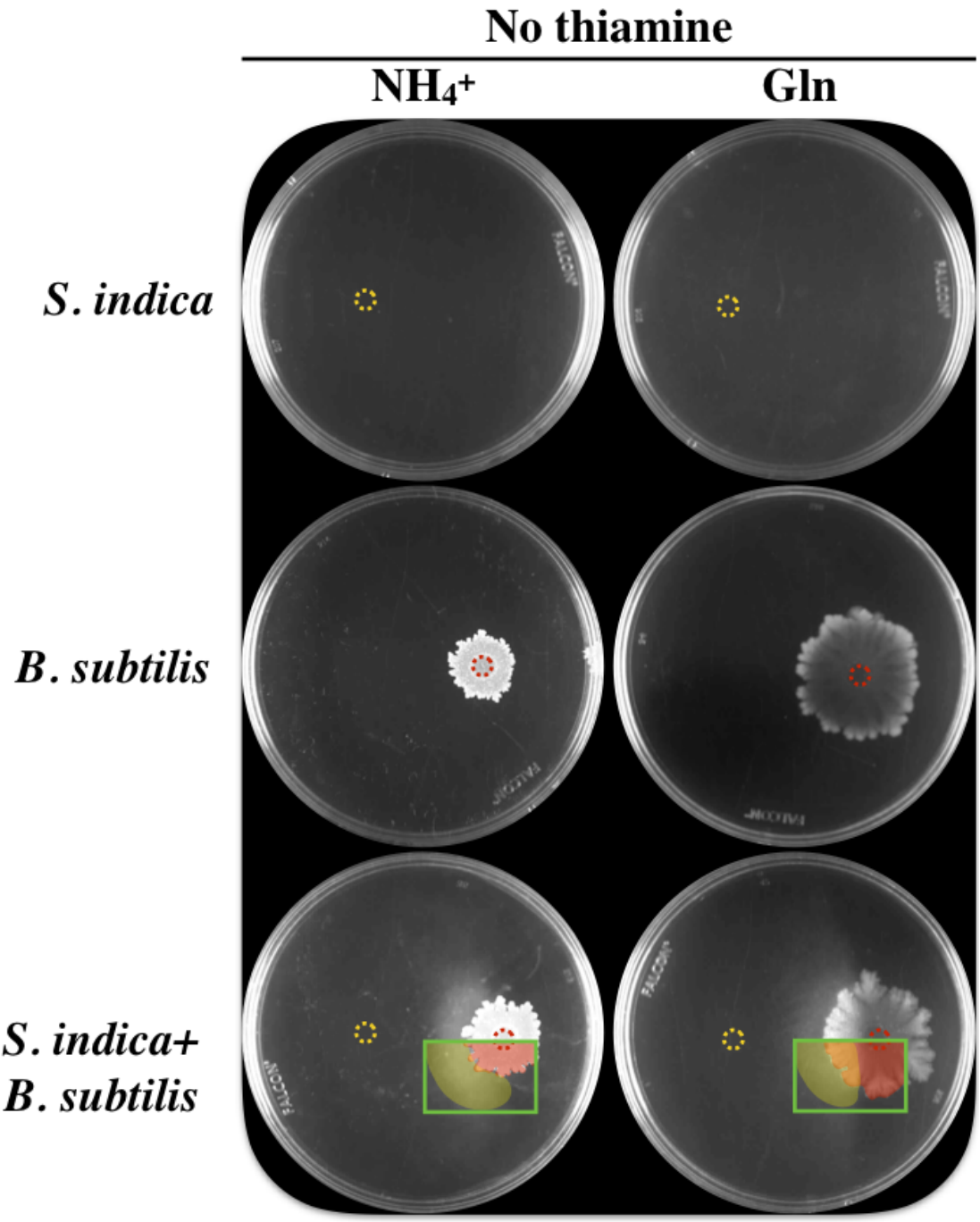


Figure 3-4: *S. indica* and *B. subtilis* growth on thiamine-free agar synthetic medium. Rows from top to bottom show growth of monocultures of *S. indica*, *B. subtilis*, and their co-culture, respectively. The left and right columns show growth on medium containing ammonium or glutamine as the sole nitrogen source, respectively. The yellow dotted circle on the images indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis* inoculation point. The green square highlights the colonies with pseudo-colour, where the area covered by *S. indica* hyphae is shown in yellow, and the *B. subtilis* colony is shown in red. The areas were manually drawn with software keynote. When both

organisms were cultured together (bottom row), *S. indica* and *B. subtilis* were inoculated on the left and right of the plate, with a 20 mm space in between. The diameter of each plate is 60 mm. Plates shown are representative of at least three replicates for each condition of two weeks' growth. Four biological replicates of this experiment were performed with qualitatively similar results.

Similar growth promoting effect was also observed in liquid cultures. *S. indica* was grown in liquid culture on its own or with supernatant from *B. subtilis* in the synthetic medium with different nitrogen source (ammonium or glutamine) and different thiamine condition (with/without) as described in method section 3.3.4. As with agar plates, in the absence of thiamine, the *S. indica* growth was limited to spore germination stage and no further growth was detected (Figure 3-5). When supplemented with *B. subtilis* supernatant, however, *S. indica* showed significantly increased growth (Figure 3-5). There was also a growth enhancement of *S. indica* by the *B. subtilis* supernatant (approximate 75 % ~ 79 % more dry weight when ammonium was the sole nitrogen source, and approximate 35 % ~ 64 % more when glutamine was the sole nitrogen source) when cultured in the presence of thiamine.

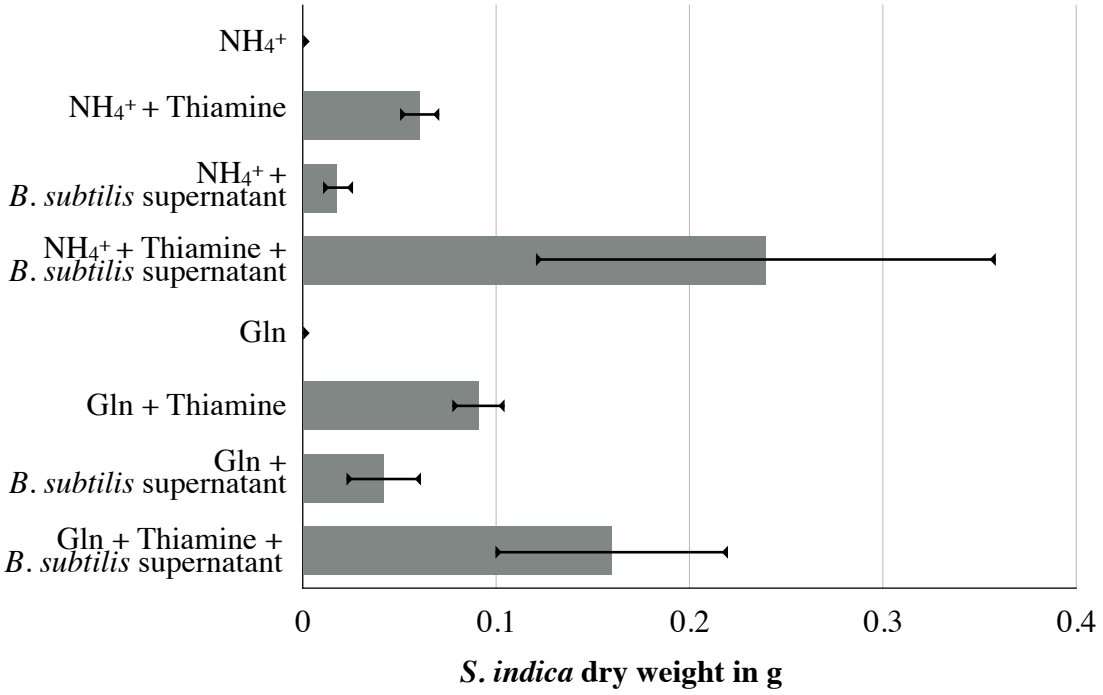


Figure 3-5: *S. indica* growth under different liquid media compositions. *S. indica* was cultivated in synthetic medium containing either ammonium or glutamine as nitrogen source, and supplemented with thiamine or *B. subtilis* supernatant. Media used for growing *S. indica* are indicated on the *y*-axis. The *x*-axis shows *S. indica* growth approximated by total dry weight after 1 week of growth. Three technical replications were used for generating the weight data and standard deviation error bars. Data shown here is from one of the two biological replications, with others giving quantitatively similar results.

While the findings (Figure 3-4 and Figure 3-5) strongly suggest that the growth-enabling of *S. indica* by *B. subtilis* was due to thiamine supply, it is theoretically possible that *B. subtilis* provides metabolites other than thiamine, which allow bypassing of central reactions requiring ThPP as a co-factor, in other words, provision of metabolites that are downstream of pyruvate in the TCA cycle (Figure 2-11). This possibility was examined with the previous experiment on *S. indica* growth screen on organic and amino acids that link to the central carbon metabolism (described in result section 2.4.3). As such, none of the 17 amino acids or 8 organic acids tested, or their combinations, allowed for *S. indica* growth in the absence of thiamine (Figure 2-7). This finding further confirmed that *B. subtilis* facilitated growth of *S. indica* in thiamine-free medium is linked directly to thiamine.

3.4.2 Metabolic profiling shows additional metabolic interactions between *S. indica* and *B. subtilis*

For a deeper understanding of the causes behind the positive impact from *B.subtilis* supernatant， and to analyse the basis of metabolic interactions between *S. indica* and *B. subtilis*, examination of the exchanged metabolites between the two organisms is crucial. Co-culture on agar plates make it difficult to extract metabolites,

therefore co-culture in a liquid medium is required for collecting exchanging metabolites. Due to *S. indica* forming clusters in liquid culture (see section 2.2), it was challenging to co-culture *S. indica* and *B. subtilis* together in the liquid medium while keeping clear growth record of each organism. Therefore, the metabolites exchanging experiment using supernatant cross-feeding was performed. As described in method section 3.3.4, *B. subtilis* was cultivated in liquid media (thiamine-free synthetic media containing glutamine or ammonium as sole nitrogen source) for one week. Its supernatant was collected and mixed with the same volume of fresh media. This supernatant-medium mixture was used to cultivate *S. indica* for one week; and *vice versa*, *S. indica* supernatant was used for cultivating *B. subtilis* following the same procedure.

Metabolites collected from the liquid culture cross-feeding experiment was carried on an ion chromatography (IC) based targeted metabolite quantification (described in method section 3.3.4). Key nitrogen compounds (ammonium, glutamine, and glutamic acid), and organic and amino acids relating to the TCA cycle (alanine, glycine, lactate, acetate, pyruvate, and formate) (Figure 2-11) in the supernatant of each organism before and after cross-cultivation were quantified.

The supernatant from *B. subtilis* monoculture contained significantly higher amounts of acetate (~ 10 mM) compared to the level of formate (~ 0.35 mM) and pyruvate (~ 0.2 mM) produced. The extracellular levels of these compounds did not change in the presence or absence of thiamine (within 10% variance) in the media (Figure 3-6 A). When *S. indica* was grown in the *B. subtilis* supernatant and in the absence of thiamine, the fungus consumed both acetate and formate and produced pyruvate (Figure 3-6 B). In the presence of both thiamine and the *B. subtilis* supernatant, the consumption of acetate and formate was also observed, but there was

also the production of lactate in addition to pyruvate (Figure 3-6 B). There were also a few peaks which could not be identified in the IC chromatograms that are altered during the cross-supernatant experiment (Figure 3-7).

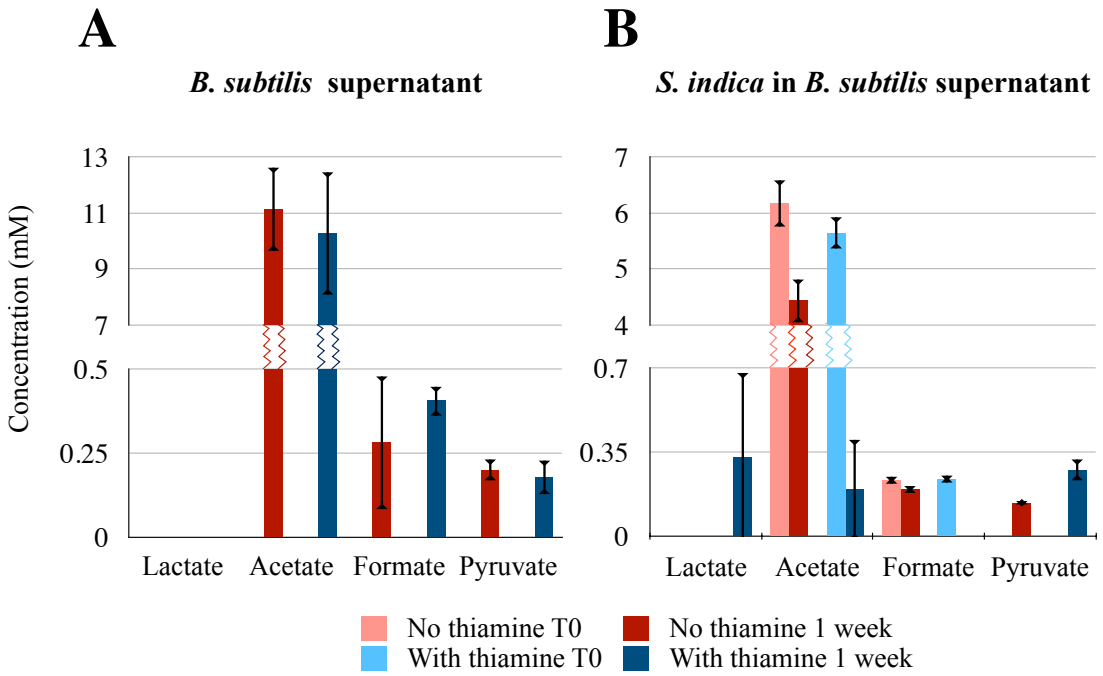


Figure 3-6: Concentrations of key organic acids in the supernatant of *B. subtilis* **(A)**, and *S. ind*ica when grown in *B. subtilis* supernatant **(B)** using synthetic medium containing glutamine as the sole nitrogen source. **(A).** The concentration of 4 organic acids accumulated in the supernatant after one week of *B. subtilis* cultivation in liquid medium containing glutamine as the sole nitrogen source. **(B).** The concentration of 4 organic acids in the supernatant after one-week cultivation of *S. indica* in *B. subtilis* supernatant mixed with the same volume of fresh medium. T0 and 1 week indicate the initial condition and after 1 week of growth respectively, while the different colours indicate the thiamine presence/absence as shown in the legend. Note that T0 concentrations in **(B)** chart correspond to concentrations in **(A)** chart diluted with same volume fresh media. Three technical replications were used for generating the concentration data and standard deviation error bars. Data shown here is from one of the two biological replications, with the other giving quantitatively similar results.

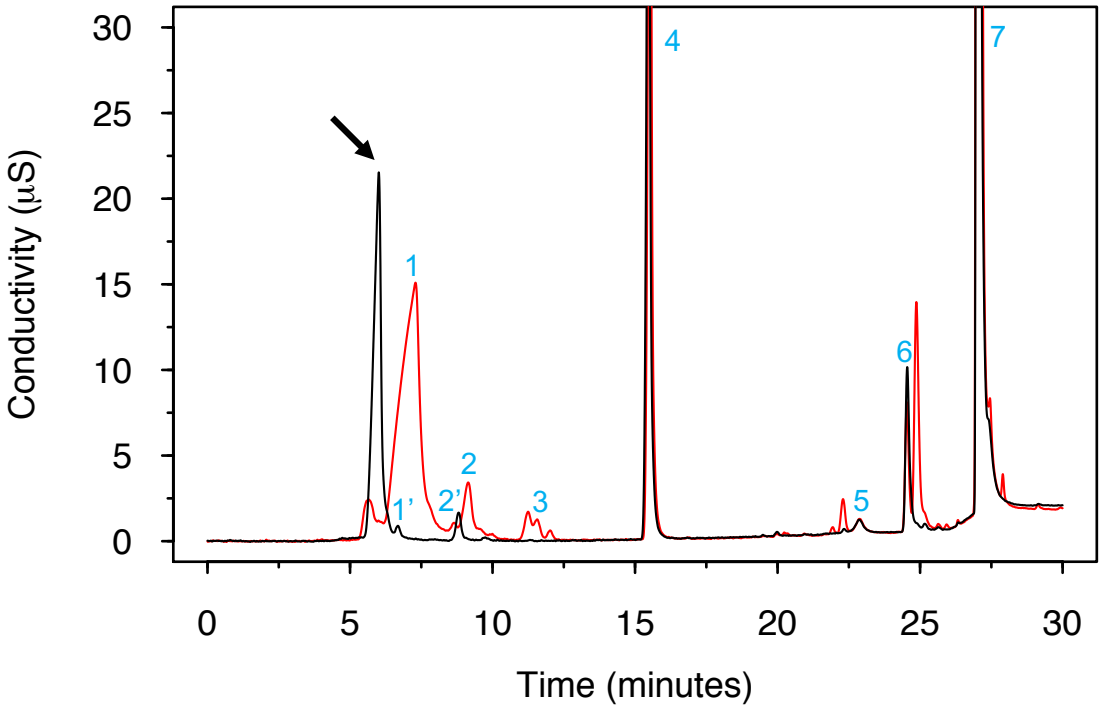


Figure 3-7: Sample chromatogram from IC analysis of cross-supernatant experiments between *S. indica* and *B. subtilis* using the synthetic medium containing glutamine as the sole nitrogen source and with thiamine. The black and red traces are for *S. indica* supernatant (diluted with an equal volume of fresh medium), and *B. subtilis* supernatant after growth in this *S. indica* supernatant, respectively. Several IC-peaks, labelled with blue numbers (with corresponding compound names given below), were identified based on retention times of known reference samples, while few other peaks remained unidentified. One prominent peak (black arrow) derived from *S. indica* culture and disappeared upon culturing *B. subtilis*. Note that some retention times are shifted because of high sample load (i.e. acetate labelled 1/1'), which also affected adjacent peaks (i.e. formate, 2/2'). Chromatograms shown are representative for a triplicate analysis. The identified peaks derive from (1) acetate, (2) formate, (3) pyruvate, (4) chloride, (5) carbonate, (6) sulphate, (7) phosphate.

In the case of the key nitrogen compounds and amino acids, in synthetic media containing glutamine as the nitrogen source, *B. subtilis* supernatant contained glutamic acid and glutamine, which were subsequently consumed by *S. indica* (Figure 3-8). In ammonium-based media, a significant amount (approximately 20 mM, close to the indicial concentration) of ammonia was left unconsumed in the *B. subtilis*

supernatant and was subsequently consumed by *S. indica*. This ammonia consumption by *S. indica* led to the accumulation of glutamic acid, glutamine, alanine, and small amounts of glycine (Figure 3-8).

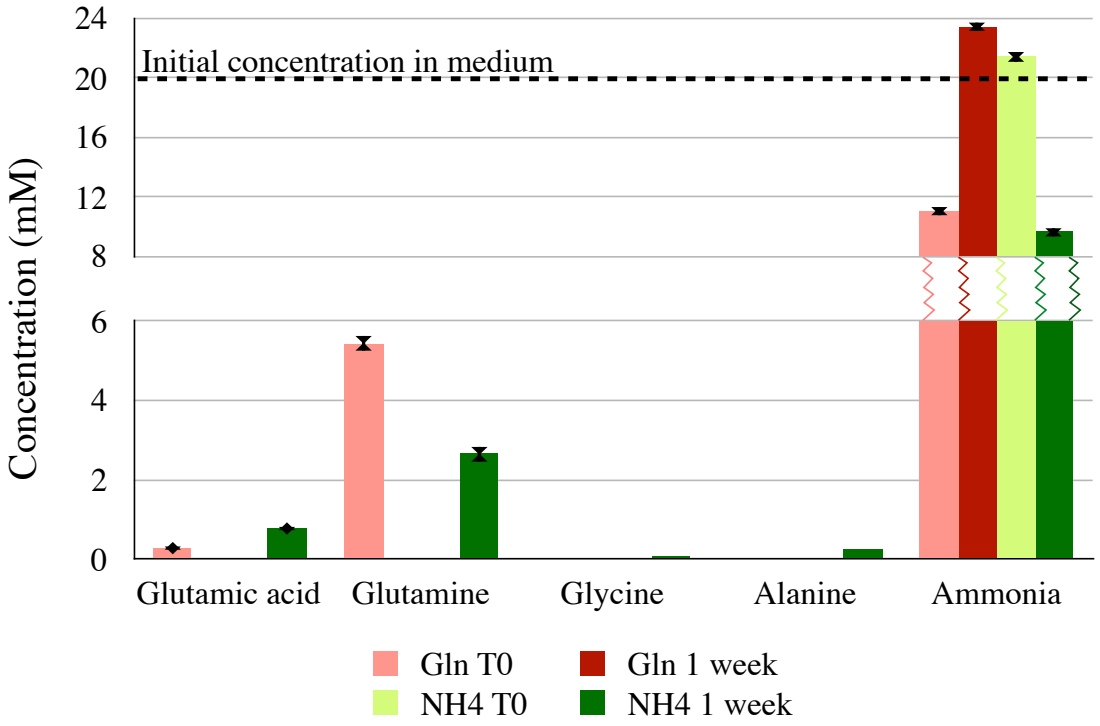


Figure 3-8: Concentrations of detected amino acids and ammonia in the supernatant of *B. subtilis* (diluted with equal volume of fresh medium), and in the supernatant after subsequent cultivation of *S. indica* using the thiamine-free synthetic medium. The labels "Gln" and "NH4" indicate the use of glutamine or ammonium as the sole nitrogen source. "Gln T0" and "NH4 T0" indicate the initial conditions, which is the mixture of supernatant from one-week *B. subtilis* culture and equal volume of fresh medium. "Gln 1 week" and "NH4 1 week" indicate the supernatant after one-week cultivation of *S. indica* in the initial condition. Black dotted line indicates the level of initial concentration of 20 mM nitrogen (glutamine or ammonium) in fresh medium. Three technical replications were used for generating the concentration data and standard deviation error bars. This amino acid analysis was performed only once due to budget limit of this study.

These findings, in particular acetate and formate cross-feeding from *B. subtilis* to *S. indica*, explain the positive impact of *B. subtilis* supernatant on growth

irrespective of thiamine availability. They also provide further support that the *B. subtilis*-associated growth of *S. indica* relates to thiamine provision rather than organic acids, since acetate and formate alone did not enable *S. indica* growth in thiamine-free media (Figure 2-7).

The detection of thiamine below 50 µg/l was unreliable with the established thiochrome-plate reader method in this study. However, thiamine from ten-times concentrated *B. subtilis* cultures could be detected. *B. subtilis* was cultivated in thiamine-free synthetic medium using glutamine as sole nitrogen source for one week. The supernatant of *B. subtilis* culture was brought to a ten-times concentration and tested. Thiamine in ten-times concentrated *B. subtilis* supernatant was approximately 75.6 µg/l, indicating that original supernatant of one week's *B. subtilis* culture contained thiamine at a concentration of 7.56 µg/l, and the supply to *S. indica* (with volume amount of *B. subtilis* supernatant and fresh medium) contained approximate 3.72 µg/l thiamine (in medium containing glutamine as sole nitrogen source) (Figure 3-9).

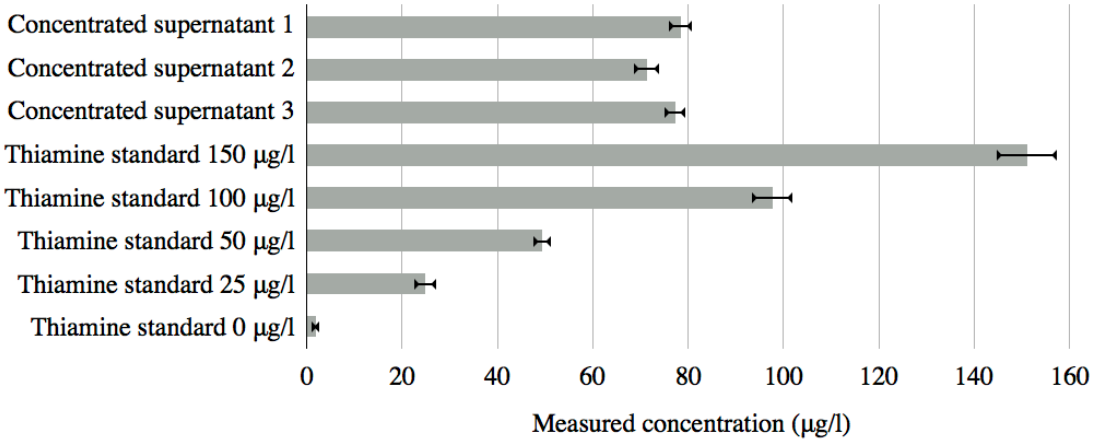


Figure 3-9: Thiamine concentration of ten-times concentrated *B. subtilis* supernatant. Three replicates of 10-times concentrated supernatant (*B. subtilis* growing in thiamine-free synthetic medium containing

glutamine as sole nitrogen source for one week) were measured together with a set of standard thiamine solutions. Three samples from each thiamine concentration and each concentrated supernatant were used for generating the concentration data and standard deviation error bars. Data shown here is from one of the two biological replications, with others giving quantitatively similar results.

3.4.3 The successful co-existence of *S. indica* and *B. subtilis* depends on the spatiotemporal organization in the system

The findings from result section 3.4.1 show that *B. subtilis* can support the growth of *S. indica* in thiamine-free medium either through its supernatant or when co-cultured at a distance on an agar plate. Both experimental setups were geared towards identifying possible interactions among the two species through utilisation of the excretions of one species by the other but did not necessarily consider the spatiotemporal factors on such interactions. Thus, the remaining question was whether both species could still co-exist and establish a successful interaction under different conditions regarding the spatial proximity or size of initial inoculation, or the actual growth phase that the different species are in at the time of introduction onto the agar. While addressing these questions is experimentally challenging, attempts have been made in this study to analyse the impact of spatial-temporal factors on the outcome of the *S. indica–B. subtilis* auxotrophic interaction by changing inoculation time and location on agar plates. In particular, the inoculation of *S. indica* spores was separated from *B. subtilis* inoculation either in time (by inoculating spores 3 days before *B. subtilis* culture inoculation) or space (by inoculating *S. indica* and *B. subtilis* at a certain distance to each other) (details were described in method section 3.3.6). Alternatively, *S. indica* spores were mixing with *B. subtilis* culture before inoculation, so that to create the non-separation scheme. These inoculation schemes mimic a scenario commonly found in agrotechnology practices when using pre-mixed cultures

or spores of different microbes as soil biofertilizers or plant seed pre-treatment (Turner, 1991).

With temporal or spatial separation, both species could successfully grow in the absence of thiamine, indicating a positive auxotrophic interaction (Figure 3-10). In contrast, direct co-inoculation of *B. subtilis* with *S. indica* significantly hampered co-existence of the two species, particularly reducing *S. indica* growth (Figure 3-10).

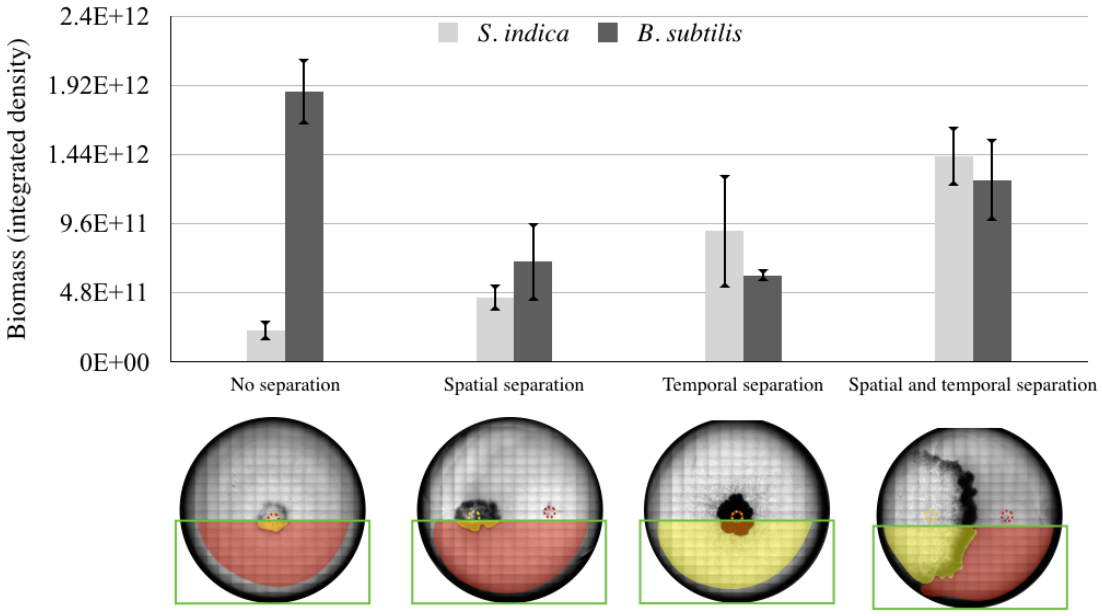


Figure 3-10: The biomass of *B. subtilis* and *S. indica* under different spatiotemporal culturing schemes. Thiamine-free synthetic medium containing ammonium as the sole nitrogen source was used. The chart shows the growth of the two species, approximated by tracing their respective colonies on the plate, and measuring the image intensity from the engulfing areas 2 weeks after *S. indica* inoculation. In this chart, biomass data and standard deviation error bars are generated from the measurement on five technical replicate agar plates. Bottom images show microscopic scans of plates at 2 weeks of growth. Each image is the representative of the five replicates under one treatment. The diameter of each image is 35 mm. "No separation" refers to *B. subtilis* culture and *S. indica* spores being pre-mixed at 1:1 volume ratio, and then inoculated as a single solution. "Spatial separation" refers to approximately 1.5 cm separation of *S. indica* (left) and *B. subtilis* (right) inoculation points. "Temporal separation" refers to inoculation of *S. indica* 3 days prior to *B. subtilis* inoculation. "Spatial and temporal

separation" refers to inoculation of *S. indica* 3 days prior to *B. subtilis* inoculation, with approximately 1.5 cm separation of *S. indica* (left) and *B. subtilis* (right) inoculation points. The yellow dotted circle on the images indicates the *S. indica* inoculation point. Red dotted circle indicates *B. subtilis* inoculation point. The pseudo-colouring highlighted by green square indicates *S. indica* hyphal area (in yellow) and *B. subtilis* colony (in red). The area of each colonies was manually drawn. For the "no separation" case, there was no observable *S. indica* colony expansion after 1 week. Two biological repetitions of this experiment were performed, with qualitatively similar results.

The above measurements were done using the thiamine-free synthetic medium containing ammonium as the sole nitrogen source. The spatiotemporal schemes were also tested with glutamine as the sole nitrogen source. The interaction pattern on ammonium and glutamine conditions were similar, that no separation resulting in no co-existence (Figure 3-11).

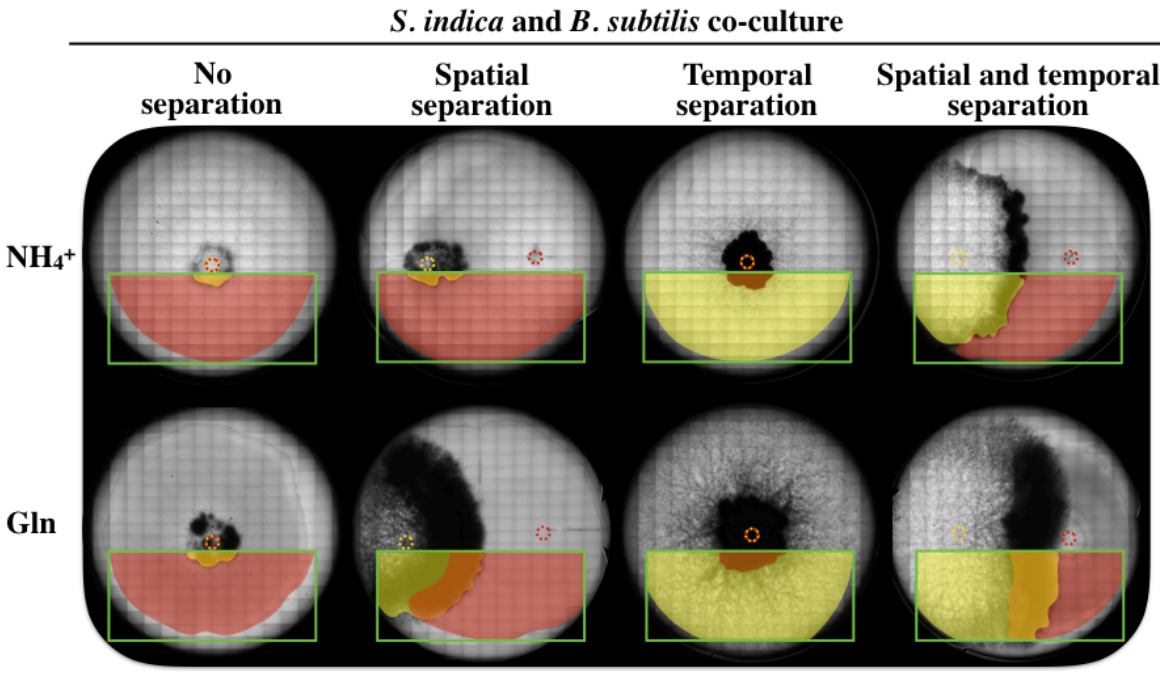


Figure 3-11: The growth of *B. subtilis* and *S. indica* co-culture under different spatiotemporal culturing schemes on synthetic medium containing different nitrogen sources. Thiamine-free synthetic media containing ammonium or glutamine as the sole nitrogen source were used. Images show microscopic scans of a representative plate out of 5 replicates at 2 weeks of growth. The diameter of each image is 35 mm. Upper and lower rows show treatments on medium containing ammonium or glutamine as the

sole nitrogen source, respectively. Columns from left to right show different inoculation schemes: "No separation" refers to *B. subtilis* culture and *S. indica* spores being pre-mixed at 1:1 volume ratio, and then inoculated as a single solution; "Spatial separation" refers to approximately 1.5 cm separation of *S. indica* (left) and *B. subtilis* (right) inoculation points; "Temporal separation" refers to inoculation of *S. indica* 3 days prior to *B. subtilis* inoculation; "Spatial and temporal separation" refers to inoculation of *S. indica* 3 days prior to *B. subtilis* inoculation, with approximately 1.5 cm separation of *S. indica* (left) and *B. subtilis* (right) inoculation points. The yellow dotted circle on the images indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis* inoculation point. The pseudo-colouring highlighted by the green square indicates *S. indica* hyphal area (in yellow) and *B. subtilis* colony (in red). The area of each colony was manually drawn. For the "no separation" case on medium containing ammonium or glutamine as the nitrogen source, there was no observable *S. indica* colony expansion after 1 week. Two biological repetitions of this experiment were performed, with qualitatively similar results.

3.4.4 Oxygen depletion could lead to the failure of colony establishment

One possible explanation for this observed strong effect of co-inoculation could be that alterations of the microenvironment by one species cause inhibitory effects on the other. Indeed, previous studies have shown that microbial growth on agar plates can create oxygen depletion within a colony (Dietrich *et al.*, 2013; Peters and Wimpenny, 1987; Kempes *et al.*, 2014). This explanation could be particularly relevant in these spatial-temporal experiments, as germination and initial growth of some soil fungi are shown to require substantial oxygen (Tacon *et al.*, 1983).

To gain insight on oxygen levels in this experimental system, the experiments shown in Figure 3-10 have been repeated, with the measurement of oxygen levels on the agar plate (see method section 3.3.7). As reported observation before (Kempes *et al.*, 2014), oxygen was completely depleted on the *B. subtilis* colonies, already within few hundred microns underneath the surface of the plate/colony (Figure 3-13). In

contrast, oxygen levels on the inoculation and growth zone of *S. indica*, as well as elsewhere on the agar plate remained high to significant depths from agar/colony surface (Figure 3-12). These results support the hypothesis that oxygen depletion by *B. subtilis* might inhibit germination of *S. indica* spores in "pre-mixed" inoculations.

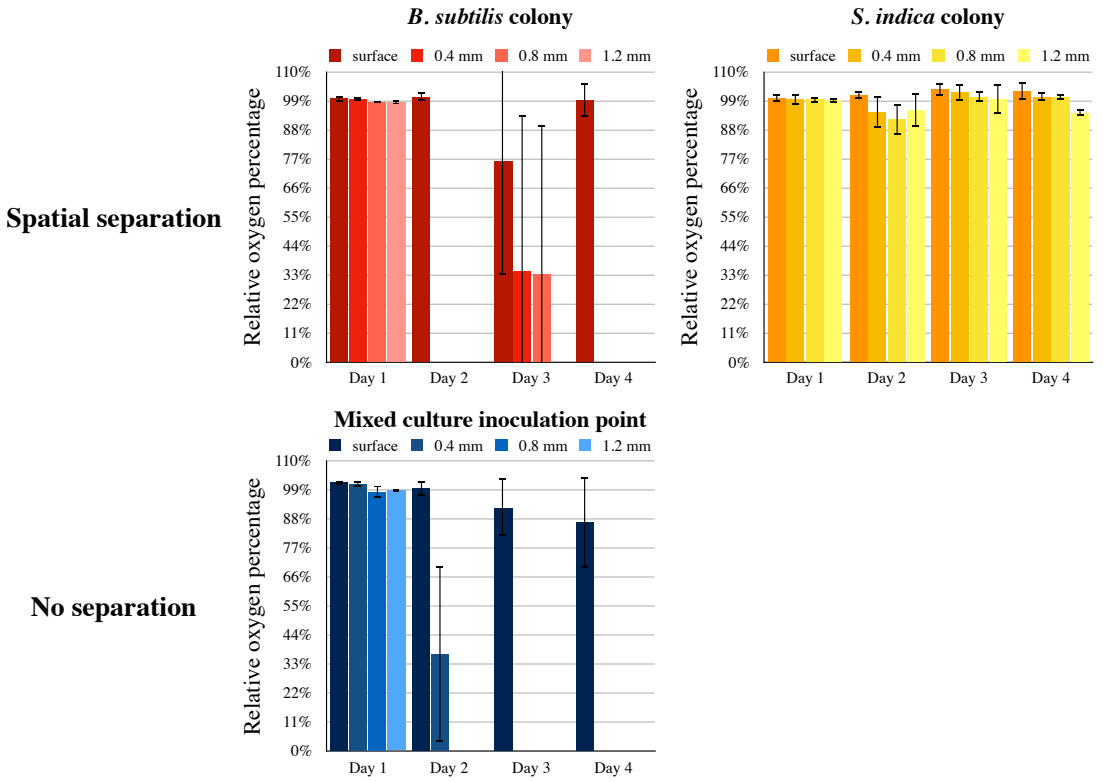


Figure 3-12: Relative oxygen percentages at different depths of plates. "No separation" refers to *B. subtilis* culture and *S. indica* spores being pre-mixed at 1:1 volume ratio, and then inoculated as a single solution. "Spatial separation" refers to approximately 1.5 cm separation of *S. indica* (left) and *B. subtilis* (right) inoculation points. "Surface" indicates the initial measurement point, which was approximately at the surface of a plate/colony. The subsequent measurements are taken at a depth of 0.4, 0.8, and 1.2 mm from this initial point, and are labelled with these values on the Figure. Oxygen percentage is shown on the Y-axis, and is normalised against the measurement made at the similar depth on the same plate but a location without any organism growth. Oxygen data and standard deviation error bars are generated from the measurement on three technical replicate agar plates. No biological replicate was done due to the time limit of this study.

Another possible reason for the observed spatiotemporal effects could be pH, which this study was only able to explore at some extent due to time limitations (see Appendix B).

3.5 Discussion

The co-culture and cross-feeding experiments showed *S. indica* growth on thiamine-free synthetic media can be restored when co-cultured with *B. subtilis* or with *B. subtilis* liquid culture supernatant. While these experiments identified additional metabolic interactions between these two species, they supported a primary and successful auxotrophic interaction between the two organisms.

Thus, this auxotrophy based co-culture in a controllable environment (the synthetic medium) creates a scenario to explore metabolic exchange between *S. indica* and *B. subtilis* and allow possible parameter adjustment such as using different nitrogen sources to simulate changing environment. This fungus-bacterium system based on auxotrophy provides insights towards understanding how ecological dynamics and evolution of auxotrophies affect community composition and assembly.

The auxotrophic interaction between *S. indica* and *B. subtilis* could only be achieved under conditions where the inoculation (and germination) of the two species were separated in time or space. This finding adds to an array of recent studies demonstrating the importance of spatiotemporal organization for the emergence and maintenances of metabolic interactions in microbial and other systems (Embree *et al.*, 2015; Momeni *et al.*, 2013; Müller *et al.*, 2014; Ratzke and Gore, 2016; Carmona-Fontaine *et al.*, 2017). In general, such effects could emerge from a multitude of factors including competition for local resources, spatial factors such as mixing and physical contact, or changes in ecological conditions caused by species' activities. For example, observations on oxygen depletion in growing bacterial colonies have been made before (Peters and Wimpenny, 1987; Dietrich *et al.*, 2013; Kempes *et al.*, 2014), as well as the idea that changing microenvironment conditions can influence the establishment and maintenance of microbial (Ponomarova *et al.*, 2017) interactions

(Embree *et al.*, 2015; Carmona-Fontaine *et al.*, 2017; Kalenitchenko *et al.*, 2015). While a detailed elimination of each possible factor might be difficult in specific cases, findings in this chapter suggests that in the case of *S. indica* and *B. subtilis*, oxygen depletion by a growing *B. subtilis* colony can cause inhibition of *S. indica* spore germination, and result in the failure of any auxotrophic interaction from establishing. This demonstrates spatiotemporal effects for the first time for these two species, which are ecologically and agriculturally relevant. Such effects are highly important for agrotechnological practice such as seed coating with *Bacillus* species (Castillo *et al.*, 2013), and soil treatment with *S. indica* (Qiang *et al.*, 2011).

Emerging studies have revealed the extent and importance of metabolic interactions within co-cultures and larger microbial communities using metabolomics approaches (Romine *et al.*, 2017; Campbell *et al.*, 2015; Ponomarova *et al.*, 2017). The focus of this chapter was on characterising the co-culture dynamics given the primary auxotrophic interaction via thiamine. Targeted metabolomics approaches were used to identify additional metabolic interactions involving key organic and amino acids related to primary metabolism (in particular the TCA cycle). These additional interactions hint to the role of overflow fluxes from central metabolism in creating new or additional metabolic interactions that can operate on top of auxotrophic interactions. It is also possible that these different types of interactions can facilitate each other's emergence and stabilisation. For example, in the case of thiamine auxotrophy, limitations in thiamine levels can decrease the rates of pyruvate and α-ketoglutarate processing through the TCA cycle, leading to diversion into amino acids such as glutamic acid, glutamine, alanine, and glycine (see Figure 2-11), which can then create new metabolic interactions. Thus, overflow metabolism and its relation to the availability of electron acceptors or co-factors could provide a

conceptual framework to understand and study microbial interactions (Braakman *et al.*, 2017; Zerfaß *et al.*, 2017). This view is also supported by recent studies indicating the role of both carbon and nitrogen overflows in the establishment of metabolic interactions (Großkopf *et al.*, 2016; Ponomarova *et al.*, 2017; Carmona-Fontaine *et al.*, 2017).

Knowing how much thiamine is secreted from *B. subtilis* would be helpful in evaluating the interactions between *S. indica* and *B. subtilis*. Due to equipment limitations, low concentration thiamine (working concentration is 150 μg/l) cannot be measured directly from samples. Attempts have been made and only thiochrome assay modified from (Hennessy and Cerecedo, 1939) on concentrated samples could give a reliable detection of thiamine produced in supernatant from *B. subtilis*. Further method development is needed for clarifying the precise thiamine amount *B. subtilis* provides to *S. indica*.

The oxygen concentration analysis suggested oxygen as a possible driver for spatial effects observed, but there could also be additional effects that need further analyses, in particular, additional impacts on the spatial organization of the two species interaction under different nitrogen sources. These different nitrogen conditions had different impacts on pH, which can also affect the two species growth and interaction. Thus, further controlled experiments need to be performed to understand these possibly intertwined effects. In Appendix B, preliminary results were presented to this end, which need to be further explored in future studies

The presented co-culture paves the way to construct more complex systems that can include additional bacteria, fungi, or plant species. This system allows the mapping of different metabolic interactions and physiological effects these species can have on each other. Towards this direction, preliminary results of interaction of

this co-culture with the model plant *Arabidopsis thaliana* were presented in Appendix C.

There is no fixed way on analysing the microbe-microbe interactions in a synthetic microbial community. Emerging studies shows the various possibilities relating to this study of using microfluidics, such as conducting *A. thaliana* media screen (Park *et al.*, 2017), cultivating fungus (Grünberger *et al.*, 2016), examining *B. subtilis* biofilm (Liu *et al.*, 2015) and analysing real-time root-microbe interactions (Massalha *et al.*, 2017). This technique could overcome the limitations encountered in this chapter (such as restricted monitoring time points, low resolution on community physiology observation and limited media screen). Therefore, microscopic analysis and microfluidics approach of studying the synthetic community were initiated, which will be described in the next chapter.

Chapter 4

Microscopic analysis of *S. indica* and *B. subtilis* interactions

The following parts of this chapter are included in the author's publication Jiang, X. *et al.*, 2018: parts of section 4.3, figure 4-9, figure 4-10, video 1, video 2, video 3 and video 4.

4.1 Abstract

Being able to see microbe interactions in real time accelerates the understanding of their morphologies and functions. The rapid development of microscopy imaging methods has revolutionised research strength to visualise microbial physiology from many angles (Weisenburger and Sandoghdar, 2015). With the help of microscopic technology, it is possible to reveal more fundamental causes to the interactions of synthetic microbial community in this study. In this chapter, *S. indica* growth was analysed at the microscopic scale, to understand how those microscopic scale features build up to the phenomenon reported in Chapters 2 and 3. microfluidics observation method on *S. indica* was also initiated, which could become a powerful tool for further analysis on the synthetic microbial community.

4.2 Introduction

The most straightforward way to study microorganisms is to observe them. Many studies have shown the importance of the microscopic level of organism interactions that help in elucidating their ecological relationships. For example, when *S. indica* colonises barley, it turns from biotrophic to saprotrophic nutrition (from living on a live host to consume dead or decaying organic material). This colonisation pattern was revealed under the microscope observation of barley cortex cells (Lahrmann *et al.*, 2013). Another example is that the fungi under *Sebacinales* order intimately associate with bacteria *Agrobacterium tumefaciens* (previous name

Rhizobium radiobacter), that this bacterium is an endosymbiont (live within of another organism in a symbiotic relationship) of *Serendipita vermifera* hyphae by transmission electron micrograph (Sharma *et al.*, 2008).

Combining microscope observations with microfluidics devices expand the research capability on microorganisms. Microfluidics is a technology that manipulates a small amount of liquid in a micrometre-scale channel. It allows for the possibility of controlling elements within the liquid in space and time (Whitesides, 2006). Researchers have used microfluidics devices to trap *B. subtilis*, and analysis *B. subtilis* biofilms properties such as cellular communication based on ion channel (Prindle *et al.*, 2015) and collective oscillation growth (Liu *et al.*, 2015). Microfluidics can also handle plant cultivation; therefore it can be applied in investigating *A. thaliana* growth in different media (Grossmann *et al.*, 2011; Park *et al.*, 2017), and in analysing *A. thaliana-B. subtilis* or *A. thaliana-E. coli* interaction (Massalha *et al.*, 2017).

There are many studies analysing the morphology of *S. indica*, reporting about its spore fluorescence (Siddhanta *et al.*, 2017), the rhythmic growth ring (a circle formed during its colony expansion due to its periodical growth)(Pham *et al.*, 2008), and the hyphae formation within plant hosts (Lahrmann *et al.*, 2013). However, morphological changes on a smaller time-scale such as in minutes or hours have less been focused, and no real-time growth of *S. indica*, or live interaction of *S. indica* with other bacteria have been reported.

In this chapter, the real-time growth of *S. indica* on agar plates and in microfluidics devices was recorded under the microscope. Live interactions of *S. indica* and *B. subtilis* were filmed, with quantitative records indicating the dynamic

changing of *S. indica* and *B. subtilis* growth. Microfluidics device was constructed for advanced imaging of *S. indica*.

4.3 Materials and methods

Analytical-grade chemicals were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA), or Fisher Scientific UK (Loughborough, UK). Gel loading tip (polypropylene, size 0.1-10 µl, Ref 11532343, Fisherbrand) and precision pipette (single channel 0.1 µl to 2.5 µl, Ref 312000001, Eppendorf research plus) were used for aiding in very precision inoculation.

4.3.1 Material preparation

S. indica spore suspension (prepared as described in section 2.3.2) and *B. subtilis* culture (prepared as described in section 3.3.2) were prepared freshly on the same day or the day before of the inoculation for each experiment.

The solid synthetic medium was prepared based on the recipe in section 2.3.1, with 1.5% final concentration agarose. Agarose low melt seaplaque (Ref H15619, MacroSieve) was used in plate experiments represented in this chapter, for a better transparency effect under microscopy.

The liquid synthetic medium was prepared as described in section 2.3.1 for experiments with microfluidics.

4.3.2 Time lapse microscopy on *S. indica* and *B. subtilis* growth

To understand the growth of *S. indica*, and the interaction between *S. indica* and *B. subtilis*, time-lapse microscopy was performed on *S. indica* and *B. subtilis* growing on solid medium. Synthetic media containing ammonium as the nitrogen source with or without thiamine were used for cultivating *S. indica* and *B. subtilis*. Media were prepared in 6 well multi-well dish (polystyrene, tissue culture flat bottom with low evaporation lid, Ref 353046, Falcon). Each well of 6 well dish contained 3

ml medium. One microliter of *S. indica* and 1 µl *B. subtilis* were inoculated on each well respectively according to the inoculation scheme indicated in Figure 4-1.

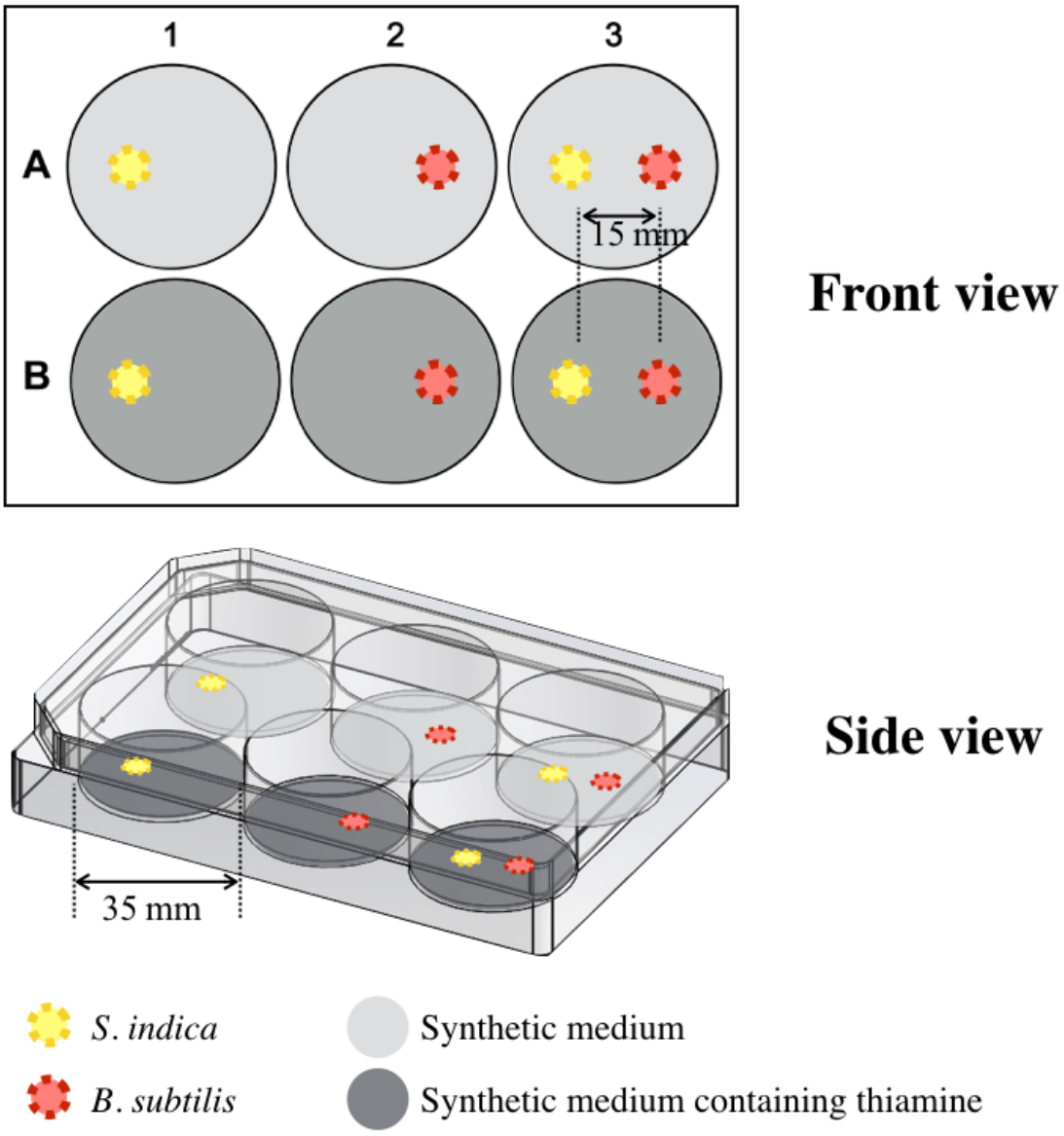


Figure 4-1: Inoculation scheme of *S. indica* and *B. subtilis* on 6-well plate for the time-lapse experiment. *S. indica* were inoculated at the left side of a well, while *B. subtilis* inoculated at the right side. When *S. indica* and *B. subtilis* were inoculated on the same well, there is a space of 15 mm in-between. A1 and B1 are for *S. indica* monoculture growth. A2 and B2 are for *B. subtilis* monoculture growth. A3 and B3 are for *S. indica* and *B. subtilis* interaction. The diameter of each well is 35 mm.

The prepared 6-well plate was sealed with parafilm and incubated in stage top incubator (H301-T-UNIT-BL-Plus system, and H301-EC chamber, Okolab) (Figure 4-2 A) at 30 ℃ for approximatly 1 week (in some cases the plates were dried out so that the experiments were terminated). The temperature sensor and lens heater of the incubator were set to 30 ℃ and stabilised for at least 2 hours prior to each experiment. This time-lapse microscopy was carried out with using Olympus IX85 microscope, Olympus UPlanFLN 4× objective (Figure 4-2 B). CellScence software was used for monitoring the experiment. Images were recorded at a 10-minutes intervals on various manually chosen locations using the bright field, phase contrast and spore autofluorescence acquisition. Spore autofluorescence was monitored at excitation/emission wavelengths of 390/470 nm.

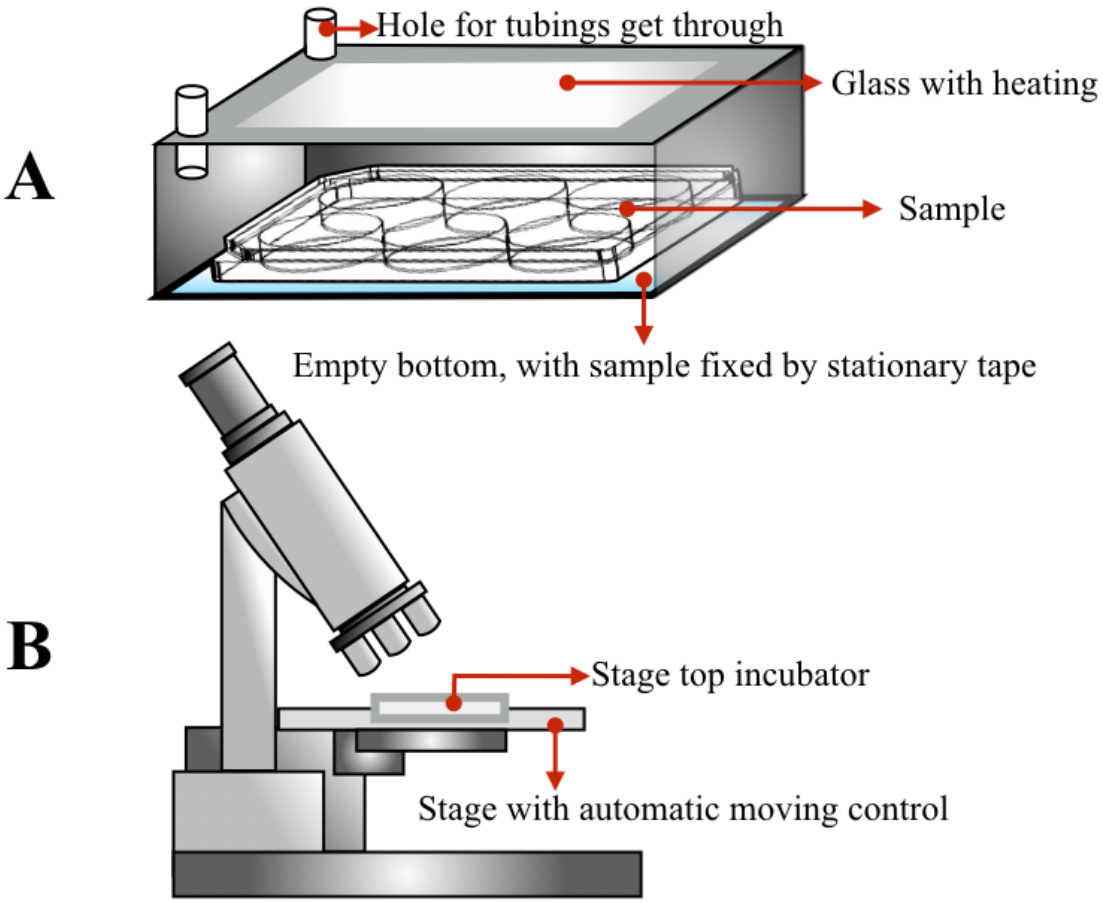


Figure 4-2: Schematic of time-lapse experiment equipment. **(A).** Stage top incubator. **(B).** Microscope with stage top incubator.

4.3.3 Image analysis on time-lapse images

Fiji (ImageJ) was used for processing the images acquired by microscope. Time-lapse images generated by Olympus CellScence software were in ".vis" form. The processed images were analysed based on the integrated intensity of manually selected locations (such as *S. indica* inoculation point, *B. subtilis* biofilm peripheral), to quantify the growth of organisms at those locations respectively.

Detailed commands performed during image processing and recorded by macros (plugin of ImageJ) are below. Words in italic font are adjustable according to need. Numbers (spreadsheets, Apple Inc.) was used for generating charts from data obtained from Fiji.

//use bioformat importer to import ".vis" files generated by Olympus system

run("Bio-Formats Importer", "open=[] autoscale color_mode=Default rois_import=[ROI manager] split_channels view=Hyperstack stack_order=XYCZT series_1");

//combile images

run("Combine...", "stack1=[*name1*] stack2=[*name2*]");

// Measure image

run("Measure...", "choose=[*name1*]");

//Then the Mean value from the pop-out result window were copied to numbers, taking //the value of first frame as blank, and calculate the rest frame by subtracting the blank value //form each : Intensity_n= Mean value_n - Mean value_1.

//Setting scale bar to image stacks, usually with 4X lens, 1 pixel equals 0.00161mm

run("Set Scale...", "distance=1 known=0.00161 unit=mm global");

run("Scale Bar...", "width=*0.5* height=*8* font=*82* color=*White* background=*Black* location=[*Lower Right*] bold overlay");

//Adding time stamp. Showing time on image stacks

setForegroundColor(*0, 0, 0*);

run("Time Stamper", "starting=0 interval=*10* x=*2220* y=*880* font=*80* '00 decimal=*-1* anti-aliased or=*min*");

//Export tiff image stack to video

run("AVI... ", "compression=*JPEG* frame=*30* save[]");

//Constructing growth indication image

run("Make Substack...", " slices=*1-(n-1)*");

run("Make Substack...", " slices=*2-n*");

imageCalculator("Subtract create stack", "Substack (*2-n*)","Substack (*1-(n-1)*)");

4.3.4 Microfluidic chamber preparation

A suitable size microfluidic chamber is required to investigate *S. indica* growth using microfluidics devices. The chamber was created by combining shaped PDMS (polydimethylsiloxane) and a glass slide. PDMS was shaped using wafer mould obtained from Dr. Raphaël Jeanneret from Physics department, University of Warwick.

The preparation procedure is as follows and illustrated in Figure 4-3. Fifteen grams in total PDMS (SYLGARD® 184 Silicone elastomer kit) were measured and mixed using a disposable cup and a plastic knife. A well-stirred mixture containing small and evenly distributed bubbles was obtained. This PDMS mixture was put in a glass vacuum desiccator (Pyrex 3121-150, Cole-parmer) connected to a vacuum pump (Maxima C, Fisherbrand) for around 20 min to remove all bubbles. A wafer mould

was covered with kitchen aluminium foil, leaving approximately 1cm by the outer side for creating a well. The centre of wafer mould was cleaned using nitrogen gas. The prepared PDMS was poured into the wafer well and heated at 90 ℃ for 30 min. The PDMS layer was peeled out the from foil. Chambers on PDMS layer were cut to the size that fits glass cover slide (35 mm × 50 mm, ref 1253E, Fisherbrand). Holes of 1mm diameter were punched on each chamber for the inlet and outlet. Dust on chambers was cleaned with stationary tape. Glass cover slides were soaked in 30% H_2O_2 and cleaned with MilliQ water and followed by ethanol. Slides were dried with nitrogen gas. Glass slides and PDMS chambers were plasma oxidised for bonding in a plasma generator device in Warwick physics department. The combined microfluidic chamber was cured (heating up to strengthen the bound) at 95 ℃ for 10 min.

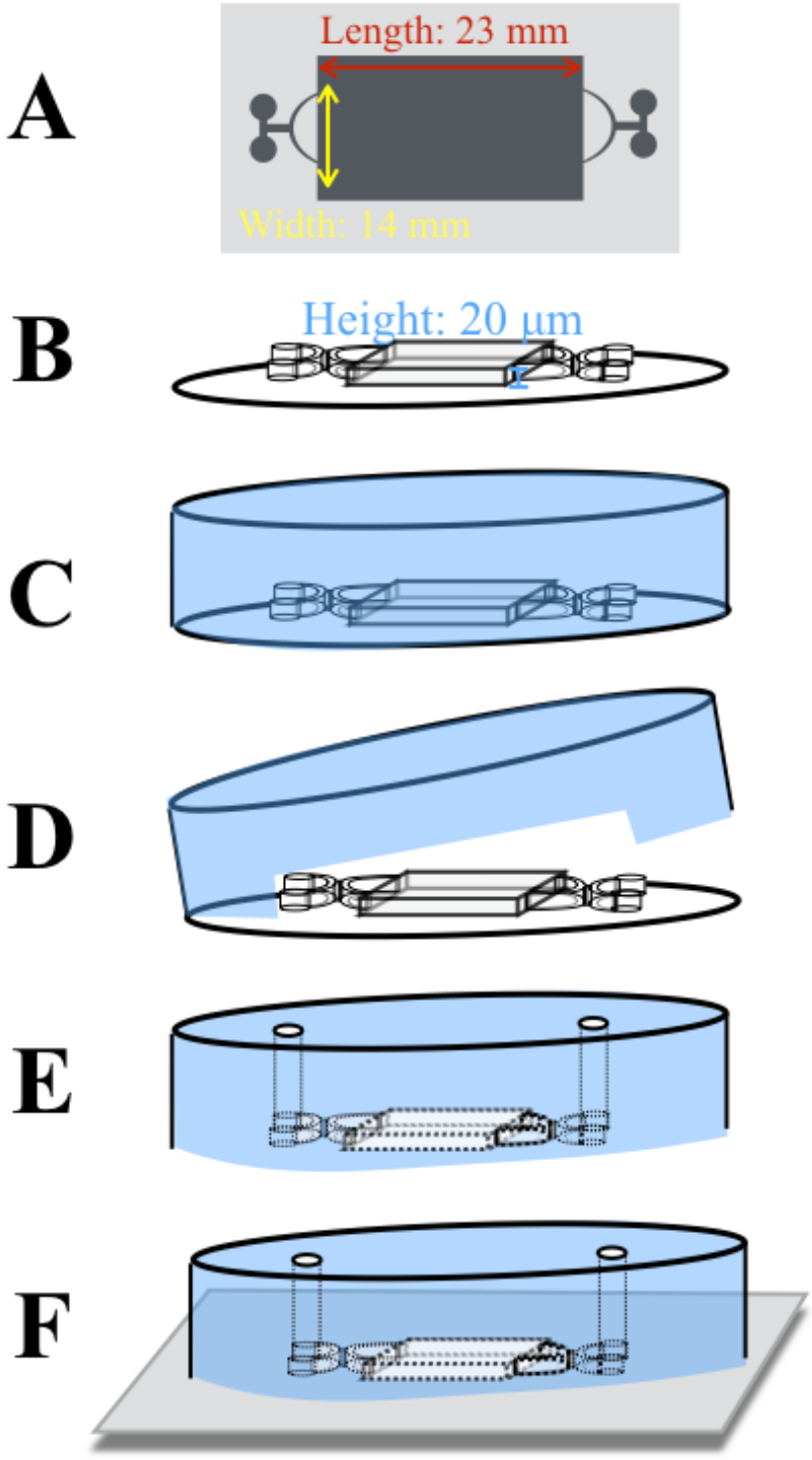


Figure 4-3: Schematic of the microfluidic chamber preparation. **(A).** Design chamber shape. **(B).** Prepare wafer mould with certain height. **(C).** Shape PDMS with wafer mould. **(D).** Peel of shaped PDMS. **(E).** Drill channel on PDMS. **(F).** Combine PDMS with a glass slide.

4.3.5 Microfluidic cultivation system set up

A whole microfluidic system was set up to cultivate *S. indica* in the microfluidic chamber. The system includes the inlet, outlet and a microfluidic chamber. The microfluidic system was combined with benchtop incubator under the microscope for observation.

Syringes (10ml and 1ml, Luer slip, Ref 302188 and 300013, BD Plastipak), metal pins cut and filed from precision dispenser needles (pink 20 gauge, Ref TE720050PK, Metcal), tubing (microbore tubing 0.020" x 0.060"OD, Ref ND-100-80, Tygon), 3-way valve (Discofix, Ref 4095111, Braun B), 50ml sterile tube (conical polypropylene centrifuge tube, Ref 352070, Falcon) were used to assemble the microfluidic system.

Metal pins were attached to tubing before inserting into the PDMS chamber. The 3-way-valve was connected to the inlet tubing by a dispenser needle. Outlet tubing was used for connecting the PDMS chamber and waste collection tube (conical polypropylene centrifuge tube, Ref 352070, Falcon). These compartments described above were put under UV light for 30 min for sterilisation. Afterwards, a syringe containing 10 ml synthetic medium (glutamine as the sole nitrogen source) with thiamine, and a syringe containing 1 ml *S. indica* spore suspension (prepared as described in section 2.3.2) were assembled to the 3-way-valve. The whole system assembly is indicated in Figure 4-4.

The whole system was put in a stage top incubator (H301-T-UNIT-BL-Plus system, and H301-EC chamber, Okolab), with the relative positions of each element fixed with stationary tape (Figure 4-2). *S. indica* spores were manually injected into the microfluidic chamber and examined under the microscope. After injecting spores, the medium was pumped into the chamber at a constant rate of 1 μl/min, controlled by

a syringe pump (infuse/withdraw programmable, Cole-Parmer). Time-lapse microscopy was carried out with using Olympus IX85 microscope, Olympus UPlanFLN 40× objective. CellScence software was used for monitoring the experiment. Images were recorded at a 10-minutes interval on various manually chosen locations using bright field and phase contrast acquisition.

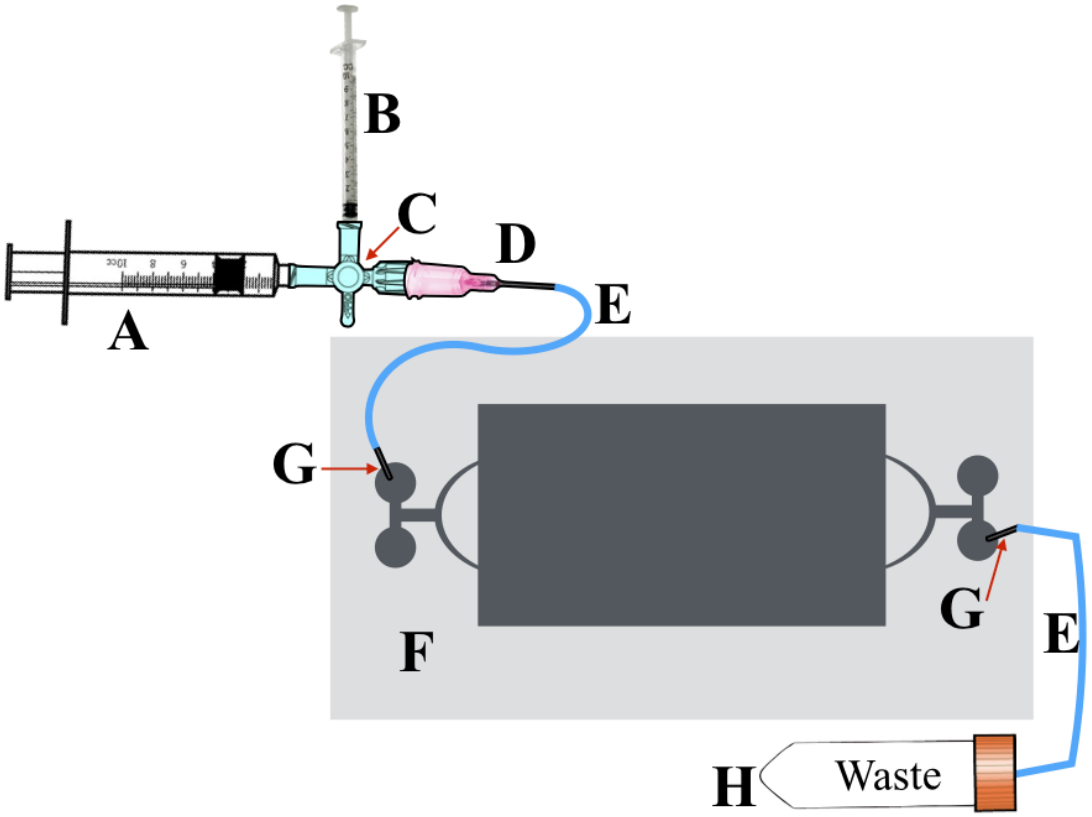


Figure 4-4: Schematic of microfluidic system assembling. **(A).** Syringe containing medium. **(B).** Syringe containing *S. indica* spore suspension. **(C).** Three-way valve. **(D).** Dispenser needle. **(E).** Tubing. **(F).** The microfluidic chamber (details see Figure 4-3). **(G).** Metal pin. **(H).** Waste collection tube.

4.4 Results

4.4.1 Time-lapse microscopy reveals different growth structures of *S. indica*

Time-lapse microscopy of *S. indica* growing on solid medium was performed to record the real-time *S. indica* growth on agar medium with or without thiamine.

In the thiamine-free synthetic medium, *S. indica* still germinated, but the growth was not constant. After approximately 40 hours, the growth under no thiamine treatment was stationary (Figure 4-6, Video 1), while under thiamine condition *S. indica* growth continually increased (Figure 4-6, Video 2). This growth status was consistent with *S. indica* growth test results previously described in section 2.4.1.

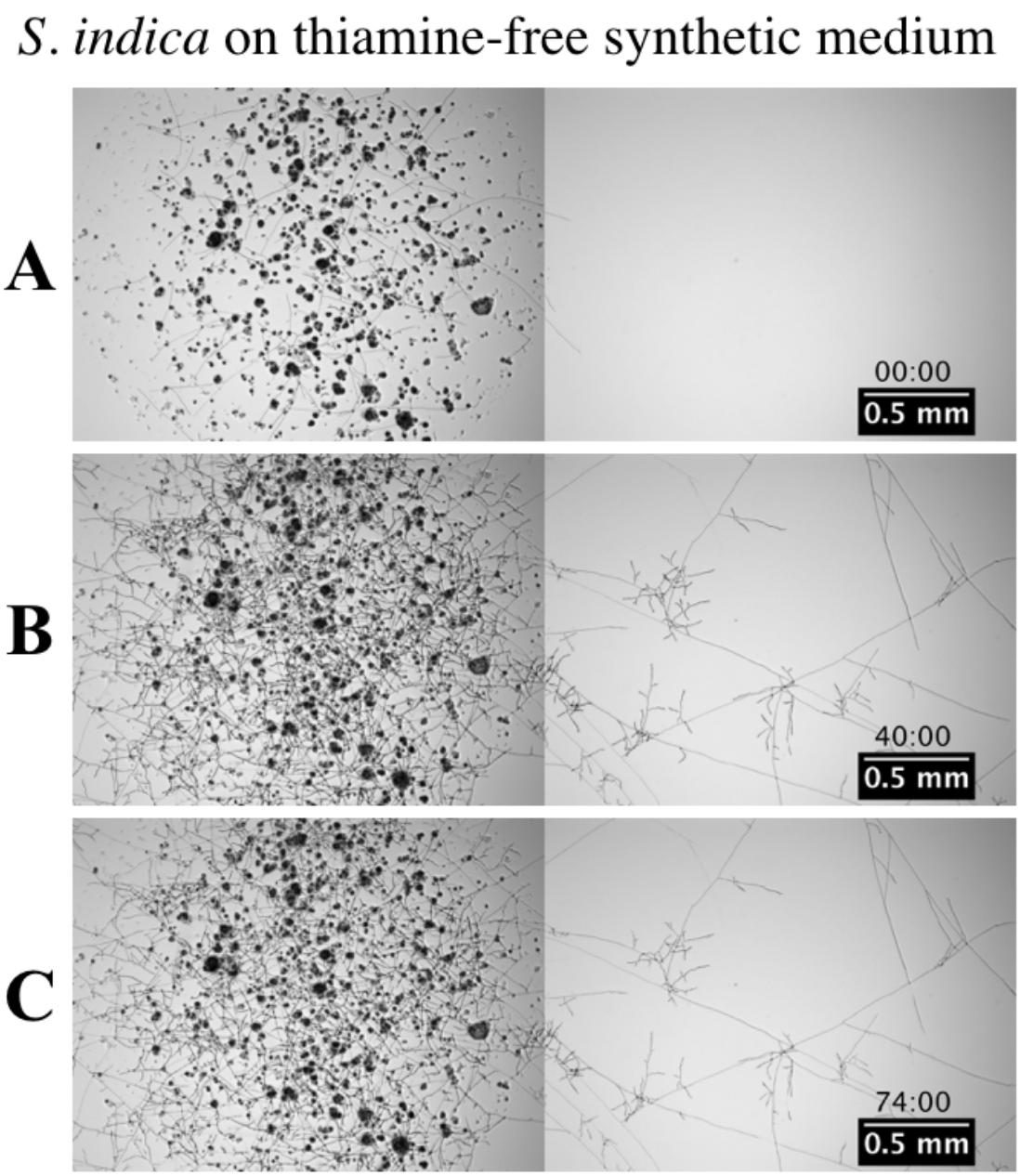


Figure 4-5: *S. indica* growth on thiamine-free synthetic medium. Images presented are the combined frames of inoculation point and one frame next to its right under the microscope. **(A)**, **(B)** and **(C)** are the growth status at 0 hour, 40 hours and 74 hours of incubation. Images presented here are a series of time-lapse images from video 1. Video 1 is the representative of at least 4 biological replicates, with others giving similar qualitative results.

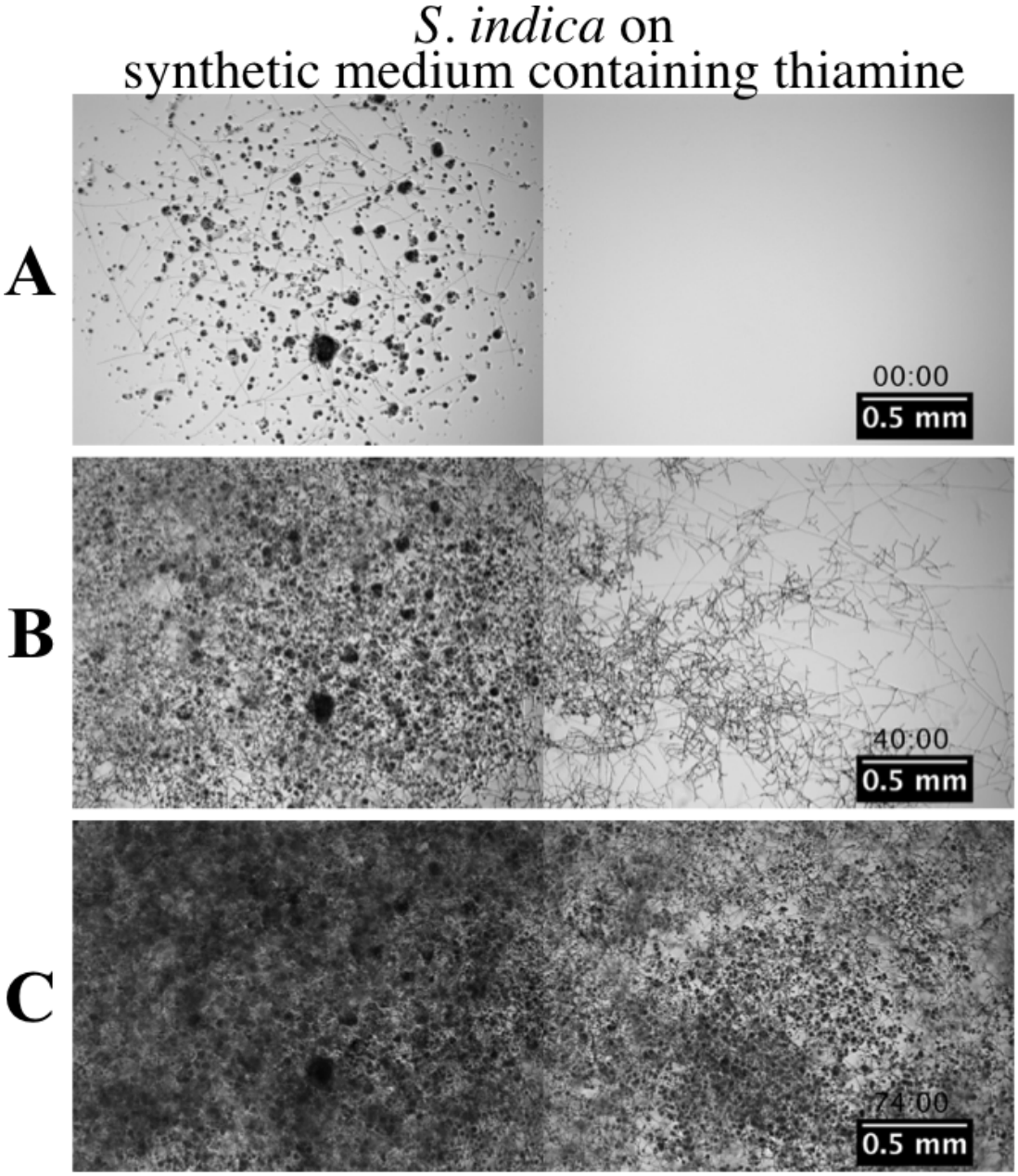


Figure 4-6: *S. indica* growth on synthetic medium containing thiamine. Images presented are the combined frames of inoculation point and one frame next to its right under the microscope. **(A)**, **(B)** and **(C)** are the growth status at 0 hour, 40 hours and 74 hours of incubation. Image presented here are a series of time-lapse images from video 2. Video 2 is the representative of at least 4 biological replicates, with others giving similar qualitative results.

In detail, when growing on thiamine-free synthetic medium, *S. indica* developed long, thick and rarely branched hyphae, usually one per spore. Those hyphae elongated quickly and unidirectionally after germinating out from spores

(Figure 4-7 A). Their features matched the runner hyphae, an architectural type defined as long, single hyphal strand with angular projections, and few or no branches, described in previous studies (Friese and Allen, 1991; Bago *et al.*, 2004). Next to the runner hyphae were some short hyphae branched dichotomously (Figure 4-7 A), similar to the branching absorbing structures (BAS) reported in previous fungi studies (Bago *et al.*, 1998; Ordoñez *et al.*, 2016). At certain time point of growth (e.g. after 40 hours) these short hyphae degenerated (Figure 4-7), that the cytoplasm retracted from hyphae tip and hyphae became empty.

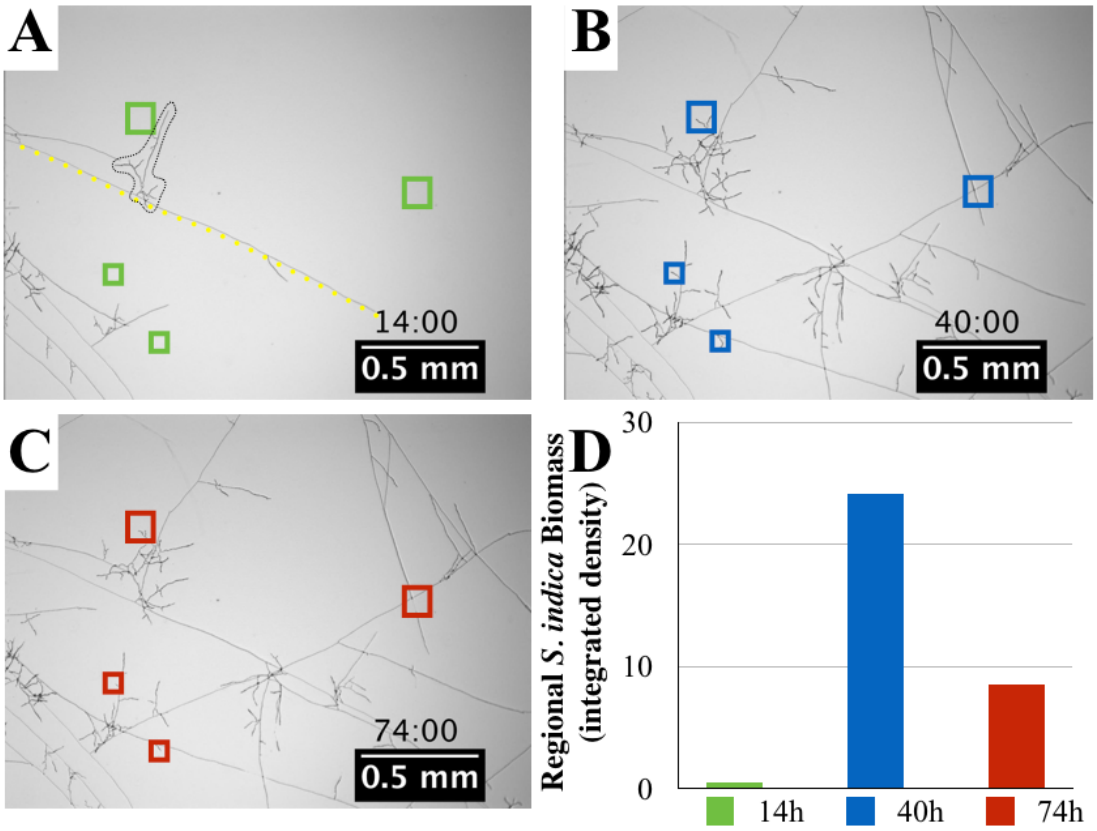


Figure 4-7: Different hyphae types of *S. indica* when growing on the thiamine-free synthetic medium. Images presented are a series of time-lapse images from video 1, at the location one frame next to inoculation point under the microscope. The yellow dotted line in **(A)** highlights the hyphae similar to runner hyphae; the black dotted circle highlights the hyphae similar to BAS (branching absorbing structure). **(A)**, **(B)** and **(C)** are the *S. indica* growth status at 14 hours, 40 hours and 74 hours of incubation. Green, blue and red squares highlight the locations where hyphae degeneration happens.

(D). Hyphae growth within the same selected region at different time points. The growth is calculated by the integrated density within all coloured squares from each time point, normalised with the integrated density of the same region at initial point (0 hour of incubation).

On the other hand, when *S. indica* grew on synthetic medium containing thiamine, small branched hyphae similar to BAS were formed after the runner hyphae developing. Compared with BAS formed on the thiamine-free medium, these hyphae did not degenerate, and produced spores after certain time (Figure 4-8). These features matched the spore-BAS (spore associated branched absorbing structure) reported in previous studies (Bago *et al.*, 1998; Bago *et al.*, 2004). The growth of these hyphae was continuous (Figure 4-8 D).

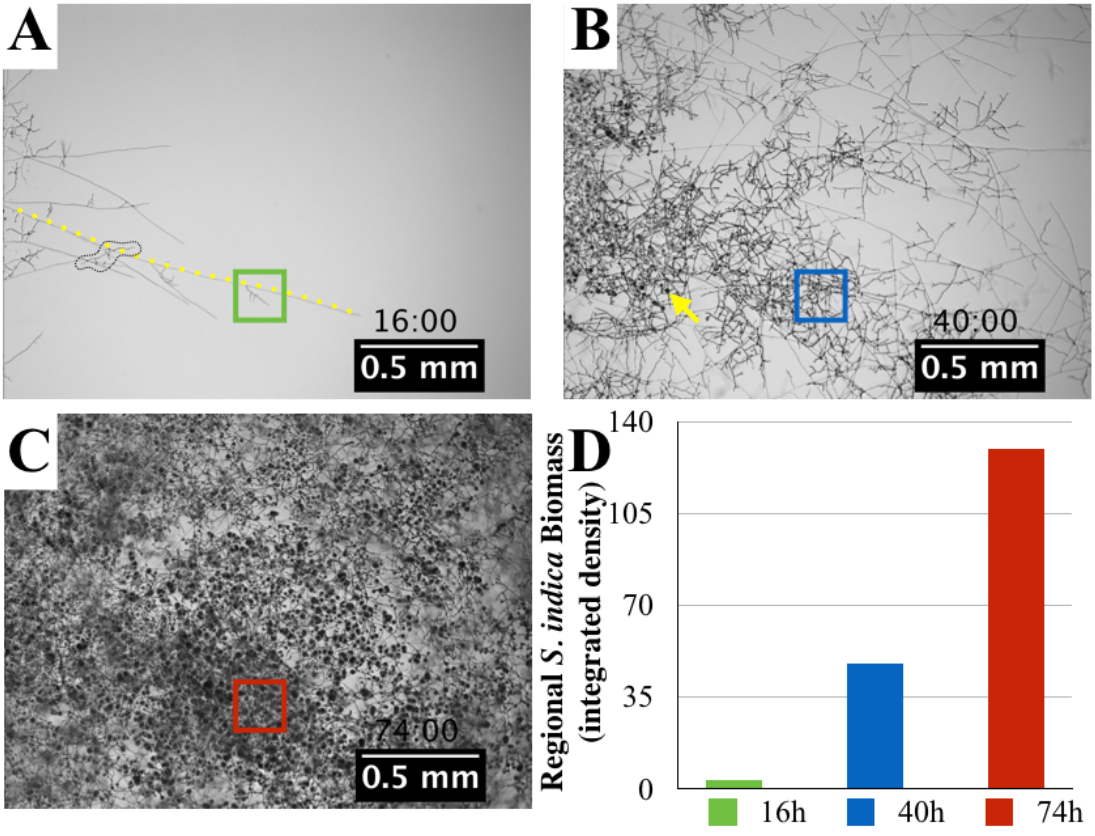


Figure 4-8: Different hyphae type of *S. indica* when growing on the synthetic medium containing thiamine. Images presented are a series of time-lapse images from video 2, at the location one frame next to inoculation point under the microscope. The yellow dotted line in **(A)** highlights the hyphae

similar to runner hyphae; the black dotted circle highlights the hyphae similar to spore-BAS (spore related branching absorbing structure). The yellow arrow in **(B)** points at one spore produced on the spore-BAS like hyphae. **(A)**, **(B)** and **(C)** are the *S. indica* growth status at 16 hours, 40 hours and 74 hours of incubation. Green, blue and red squares highlight the location where spore-BAS like hyphae formed. **(D)**. Hyphae growth within the same selected region at different time points. The growth is calculated by the integrated density within the coloured square from each time point, normalised with the integrated density of the same region at initial point (0 hour of incubation).

4.4.2 *B. subtilis* affects *S. indica* hyphae morphology and growth speed in co-culture

Time-lapse microscopy was used to quantify the spatial growth pattern of *S. indica* when co-cultured with *B. subtilis* on synthetic medium with or without thiamine and containing ammonium as the nitrogen source.

When co-cultured with *B. subtilis*, *S. indica* showed different behaviours, related to both hyphae formation and growth speed. On the thiamine-free medium, *S. indica* grew towards *B. subtilis* colony and formed a dense layer of hyphae around *B. subtilis* colony; while the side of *S. indica* colony far from *B. subtilis* was barely visible on the plate (Figure 4-9 A). The growth (as approximated by image density) happened faster at the side closer to the *B. subtilis* colony compared to the far side to *B. subtilis* (Figure 4-9 B, Video 3). The hyphae at the side closer to *B. subtilis* were mostly BAS, while the hyphae at the far side to *B. subtilis* were mainly runner hyphae (Figure 4-9 E).

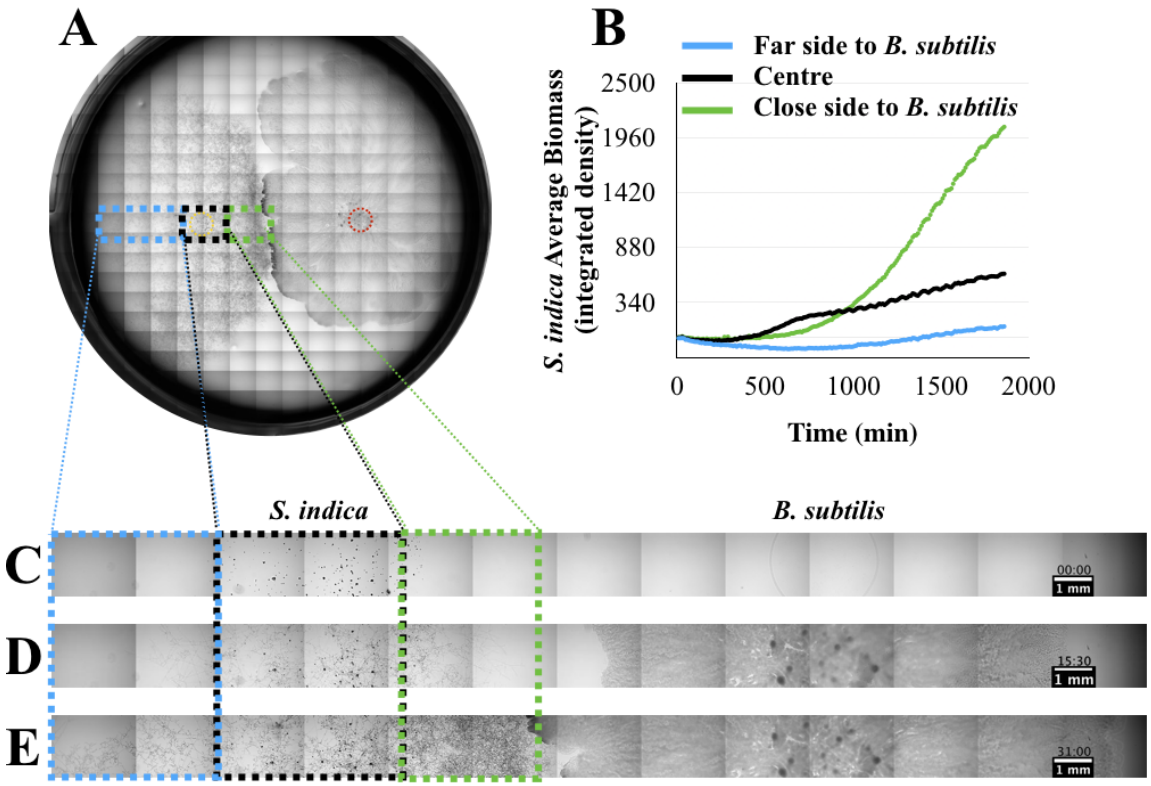


Figure 4-9: *S. indica* and *B. subtilis* co-culture on thiamine-free synthetic medium. *S. indica* colony was manually divided into three defined horizontal sections for analysis: far side to *B. subtilis* colony, centre and close side to *B. subtilis* colony, highlighted with blue, black and green dotted lines respectively. **(A).** Plate overview of *S. indica* and *B. subtilis* co-culture. This image is generated by stitching together frames of microscope-scanned images of the whole plate. The yellow dotted circle on the image indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis* inoculation point. **(B).** *S. indica* biomass obtained from the defined horizontal sections of the plate shown in **(A)**. Average biomass was calculated by integrated density of each horizontal sections, and averaged by the area of the selected section, based on Fiji image analysis. The blue, black, and green lines correspond to the growth of the horizontal sections as shown on the plate and in the time-lapse image series in **(C)**, **(D)** and **(E)**. **(C-E).** Time-lapse microscopy images from the middle cross-section of the plate through the inoculation point of *S. indica* and *B. subtilis*, at 0, 15.5, and 31 hours after inoculation. The blue, black, and green highlighted sections correspond to the *S. indica* colony side far from *B. subtilis*, the middle of the colony, and the colony side closer to *B. subtilis*, respectively. These time-lapse images presented here are a series of time-lapse images from video 3. Video 3 is the representative of at least 4 biological replicates, with others giving similar qualitative results.

On the synthetic medium containing thiamine, *S. indica* hyphae growth was similar on both sides of *S. indica* inoculation point. Consistent with the result shown in section 3.4.3, on synthetic medium containing ammonium as the sole nitrogen source, *S. indica* expansion avoided *B. subtilis* colony (Figure 4-10 A). When *S. indica* and *B. subtilis* colonies approaching each other, the hyphae growth speed decreased on the *S. indica* side close to *B. subtilis*, while on the other side hyphae continued developing (Figure 4-10 B). Compared with the hyphae at the far side to *B. subtilis* colony, most *S. indica* hyphae close to *B. subtilis* colony displayed less branching and the BAS structure produced fewer spores, (Figure 4-10 E).

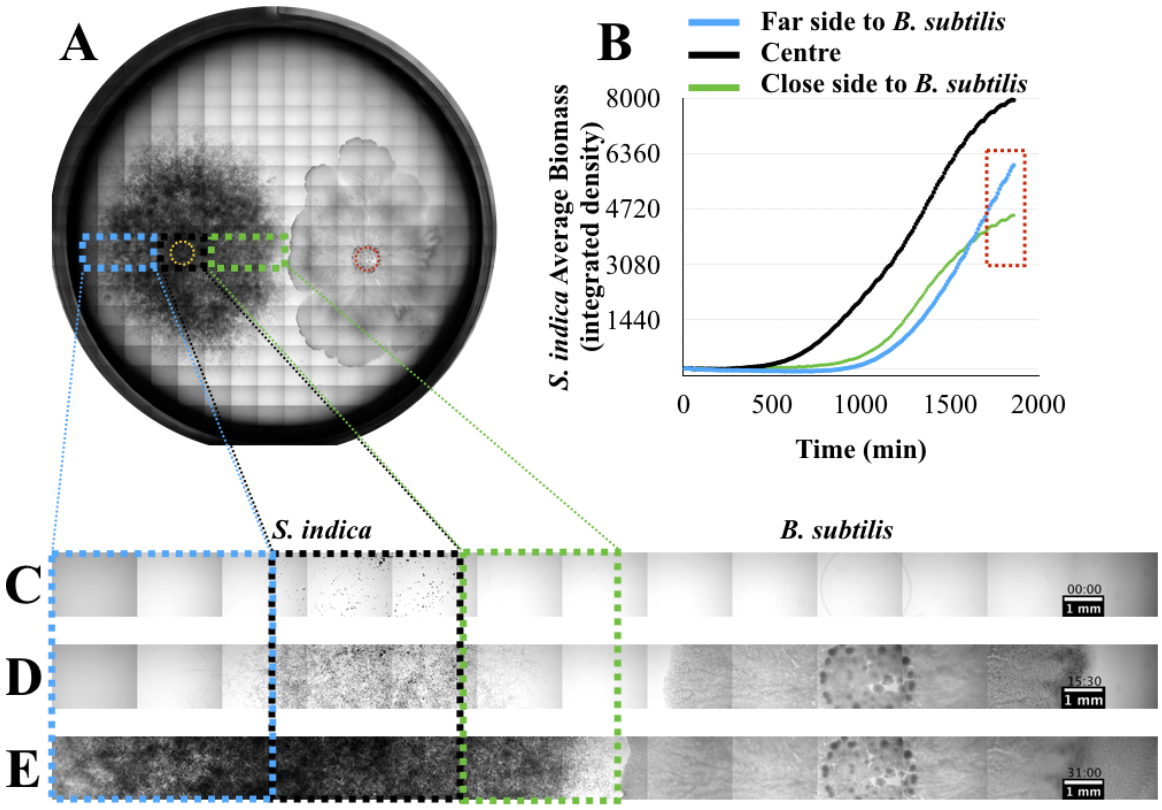


Figure 4-10: *S. indica* and *B. subtilis* co-culture on synthetic medium containing thiamine. *S. indica* colony was manually divided into three defined horizontal sections for analysis: far side to *B. subtilis* colony, centre and close side to *B. subtilis* colony, highlighted with blue, black and green dotted lines respectively. **(A).** Plate overview of *S. indica* and *B. subtilis* co-culture. This image is generated by stitching together frames of microscope-scanned images of the whole plate. The yellow dotted circle on the image indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis*

inoculation point. **(B).** *S. indica* biomass obtained from the defined horizontal sections of the plate shown in **(A)**. Average biomass was calculated by integrated density of each horizontal sections, and averaged by the area of the selected section, based on Fiji image analysis. The blue, black, and green lines correspond to the growth of horizontal sections as shown on the plate and in the time-lapse image series in **(C)**, **(D)** and **(E)**. In the red dotted square region, the slop of green line becomes flat, indicating the *S. indica* growth speed at the side close to *B. subtilis* colony is slowing down; while the slop of blue line keeps steep, indicating the *S. indica* growth speed at the side far from *B. subtilis* colony is still fast. **(C-E).** Time-lapse microscopy images from the middle cross-section of the plate through the inoculation point of *S. indica* and *B. subtilis*, at 0, 15.5, and 31 hours after inoculation. The blue, black, and green highlighted sections correspond to the *S. indica* colony side far from *B. subtilis*, the middle of the colony, and the colony side closer to *B. subtilis*, respectively. These time-lapse images presented here are a series of time-lapse images from video 4. Video 4 is the representative of at least 4 biological replicates, with others giving similar qualitative results.

The interaction patterns of *S. indica* and *B. subtilis* were mature after 2 weeks on 6-well plate (Ref: 353046, Falcon) or 60mm petri dish (Ref: 1007, Corning). On the synthetic medium containing ammonium as the sole nitrogen source, *S. indica* colony overlapped with *B. subtilis* when there was no thiamine; and *S. indica* expanded avoiding *B. subtilis* when thiamine was in the medium. These results were further confirmed by microscope observations.

After 4 weeks on thiamine-free synthetic medium (ammonium as the sole nitrogen source), *S. indica* hyphae covered *B. subtilis* (Figure 4-11 B) and reached the side of *B. subtilis* colony far from *S. indica* (Figure 4-11 C). On the other hand, on synthetic medium containing thiamine, *S. indica* hyphae stopped expanding towards the *B. subtilis* colony (Figure 4-11 E). No hypha was observed at the side of *B. subtilis* colony far from *S. indica* (Figure 4-11 F).

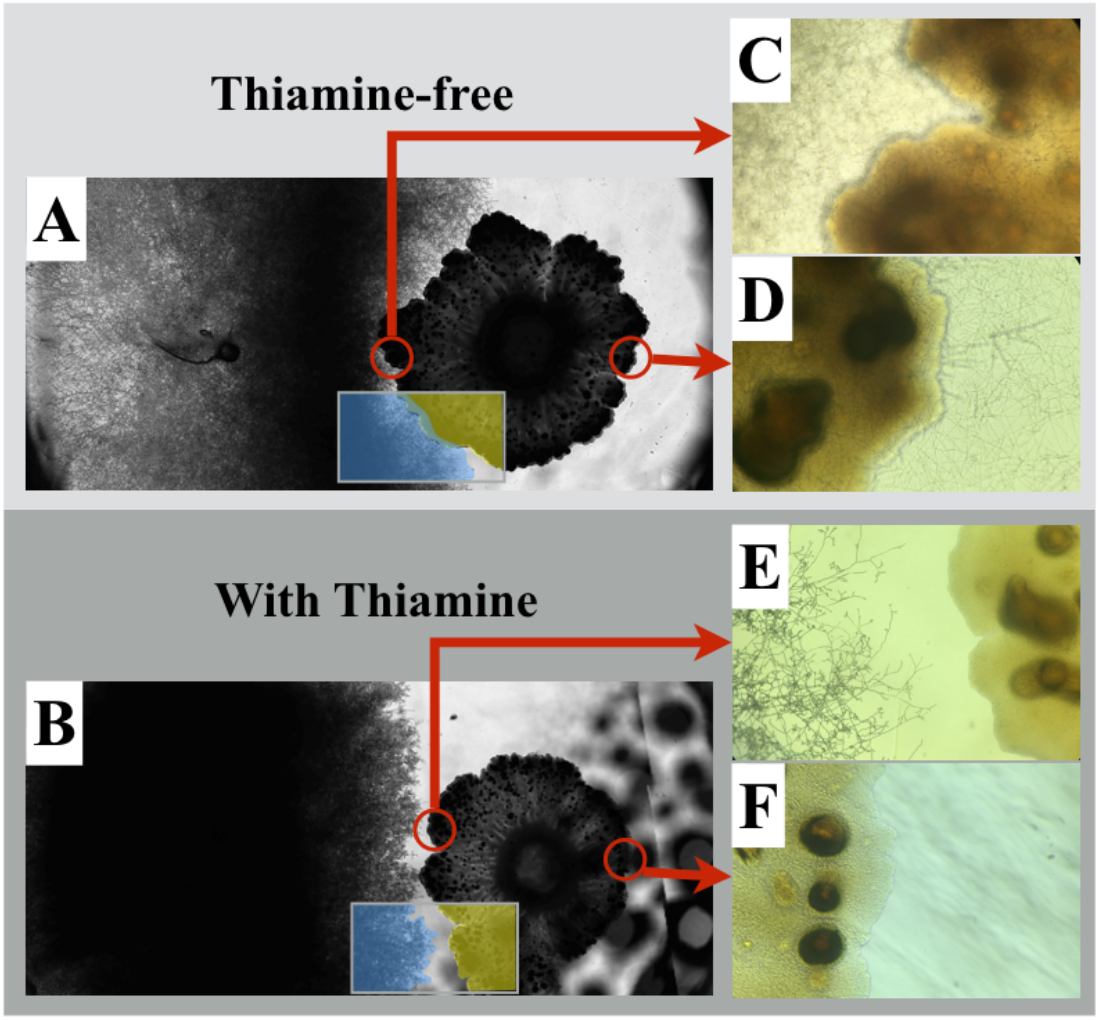


Figure 4-11: Two interaction patterns of *S. indica* and *B. subtilis* co-culture. Images shown here are co-culture on synthetic medium containing ammonium as the sole nitrogen source, with thiamine (**ACD**) and without thiamine (**BEF**). **(A)** and **(B)** are microscopic images of co-culture after 2 weeks' incubation, taken by Olympus microscope. Grey square highlights the colonies with pseudo-colour, where the area covered by *S. indica* hyphae is shown in blue, and the *B. subtilis* colony is shown in yellow. The areas were manually drawn with software keynote. **(C-F)** are microscopic images of co-culture after 4 weeks' incubation, taken by Nikon microscope. They are zoomed-in details of locations on **(A)** and **(D)** indicated with red circles and arrows respectively. *S. indica* hyphae are dark filaments; *B. subtilis* colonies are irregular brown shapes with dark brown dots. Images shown here are representative of at least 4 biological replications, with others giving qualitatively similar results.

4.4.3 Microfluidic system for recording *S. indica* growth

A microfluidic system was built for recording *S. indica* growth, allowing for observation at higher magnification such as 400 times. The microfluidic chamber (Figure 4-12) was approximate 20 μm in height to hold *S. indica* spores, whose length and diameter were of the similar scale (Kost and Rexer, 2013).

The real-time germination process of *S. indica* spore was captured using the microfluidic system (Figure 4-13, Video 5). In the newly formed hypha, particles were forming inside hyphae and merged sequentially.

The branching of *S. indica* hyphae was also recorded using the microfluidic system. However, this was only possible under lower magnification (40 times) due to equipment limitation. A preliminary result was obtained and reveals the real-time growth and branching of *S. indica* (Figure 4-14, Video 6).

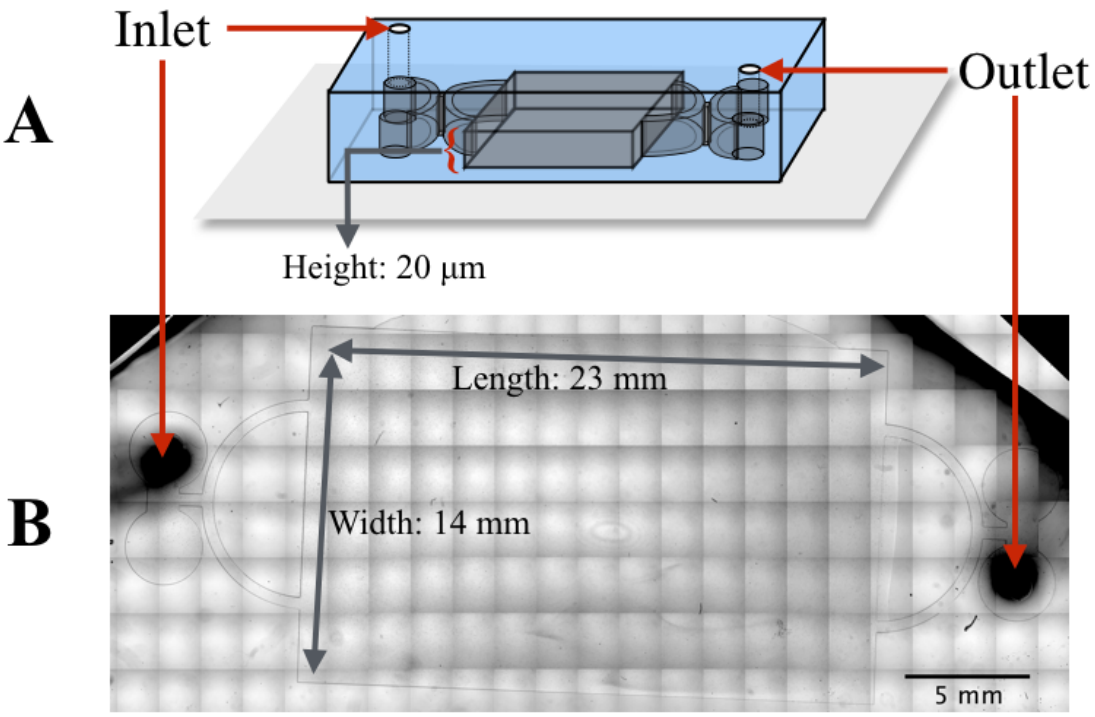


Figure 4-12: Overview of the microfluidic chamber. Chamber dimension is 23 mm × 14 mm × 0.02 mm (length × width × height). **(A).** Three-dimensional schematic of the microfluidic chamber. Blue

region represents the PDMS. Grey structure represents the chamber, which is also the hollow part inside PDMS. **(B).** Microscopic view of the microfluidic chamber. The presented image is a combination of frames scanned under the microscope using 40 times magnification of the whole chamber area. Medium entered the chamber from the inlet and exit through the outlet. Inlet and outlet positions are pointed with red arrow.

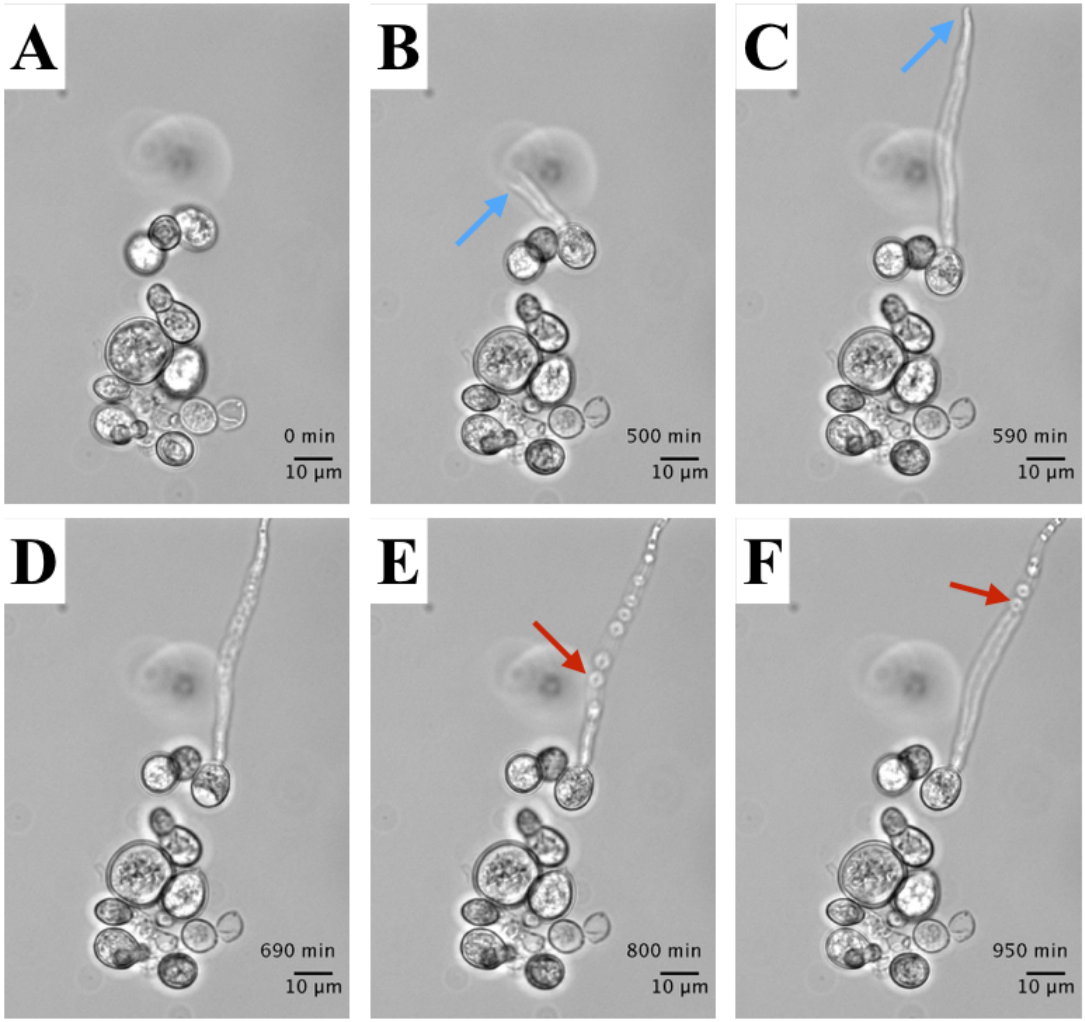


Figure 4-13: *S. indica* spore germination process. Blue arrows point to the tip of newly germinated hypha. Red arrows point to the same particle formed within hypha. **(A).** The initial status of spores. **(B).** Hypha germinates from a spore. **(C).** The hypha continues elongation. **(D).** Particles begin to form inside the hypha. **(E).** Clear round-shaped particles formed inside the hypha. **(F).** Particles sequentially merge, beginning from the spore side. These time-lapse images presented here are a series of time-lapse images from video 5. Video 5 is the representative of at least five spores' germination among 2 biological replicates, with others giving similar germination phenomena.

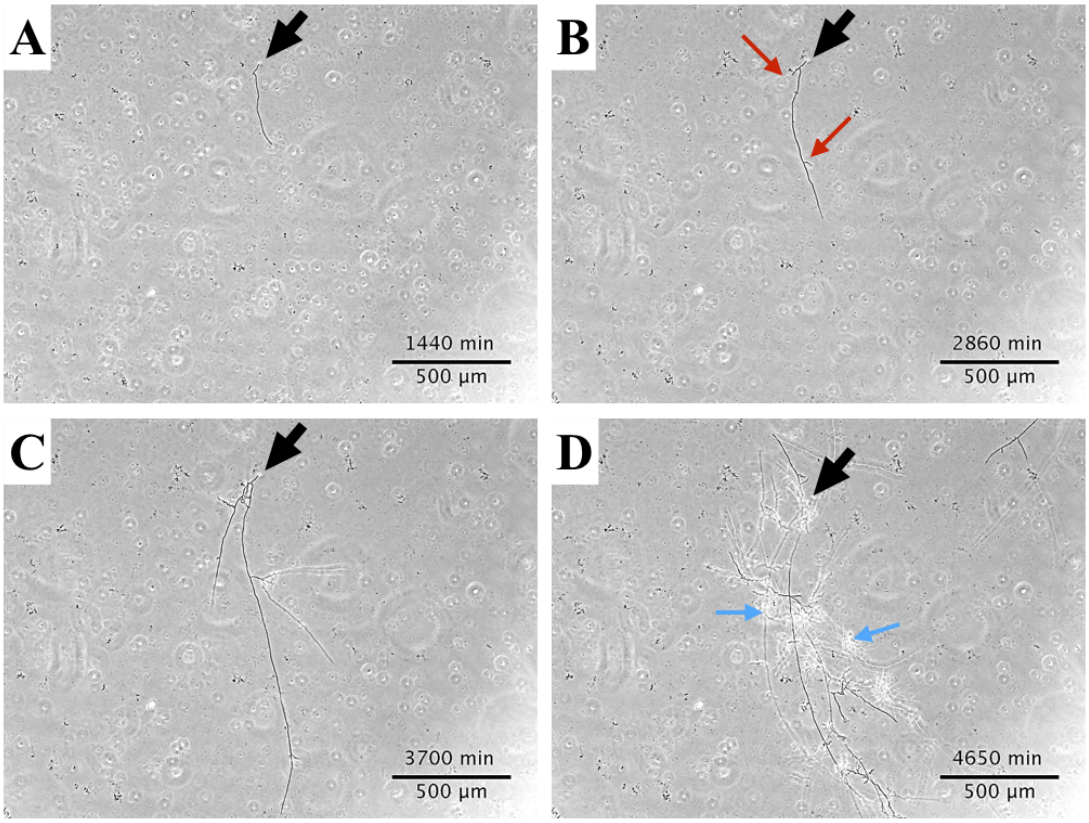


Figure 4-14: *S. indica* hyphae branching process. Black arrows point to the single germinating spore that produce hyphae. Red arrows point to the first branching point of the hypha. Blue arrows point to the hyphae parts similar to BAS (branched absorbing structure). **(A-D)** are the hyphae status at different incubation time. **(A).** Runner hypha germinates from spore, appears as dark line. (B). First branching begins. (C). More branching happens while hyphae elongating. Hyphae appear as dark or white lines, due to some of them being out of focus. (D). As more hyphae produced, the original spore has been dragged away from its original position. BAS structures appear as white filaments, due to them being out of focus. These time-lapse images presented here are a series of time-lapse images from video 6. Video 6 is the representative of four hyphae found in one experiment. No biological replicates were performed due to equipment limitation.

4.5 Discussion

The time-lapse microscopy of *S. indica* on solid medium highlighted the detailed growth of *S. indica* hyphae, and further supported the discovery of *S. indica* thiamine auxotrophy. This is the first report on real-time *S. indica* growth at a scale of tens of minutes, yielding quantifiable results for growth calculation.

Different hyphae structures were observed during *S. indica* colony development: runner hyphae, BAS and spore-BAS. The function of *S. indica* runner hypha was not made clear by this study, as it was produced on both media conditions (with or without thiamine). On the other hand, the BAS produced on thiamine-free medium and spore-BAS produced on medium containing thiamine are consistent with other studies reporting about these structures relating to nutrient-limited (BAS formation) and nutrient-rich environment (spore-BAS formation) respectively (Bago *et al.*, 1998; Bago *et al.*, 2004). The BAS effectively occupies the surface of medium, making it easier to absorb nutrition. On the thiamine-free medium, BAS degenerated, possibly due to *S. indica* experiencing nutrient deficiency. The loss of those BAS hyphae in the poor-nutrient area might reduce the cell maintaining costs in such situations. On medium containing thiamine, spores were produced on BAS structures. Since BAS relates to nutrient absorbing function (Bago *et al.*, 2004), these spore-BAS might have switched from assimilative to reproductive metabolism for optimal growth conditions.

The time-lapse microscopy allowed for the elucidation of *S. indica* and *B. subtilis* interactions by presenting real-time changing of colony structures and provided quantitative information on growth speed of different regions on colonies. The real-time interaction of *S. indica* and *B. subtilis* co-culture in this chapter further supported the results reported in section 3.4.1 and B.3.1, that *B. subtilis* can support *S.*

indica thiamine auxotrophy, and *S. indica* and *B. subtilis* have different interaction patterns.

On thiamine-free medium, *S. indica* growth speed increased at the side close to *B. subtilis*. This could be explained by the presence of an increasing thiamine gradient towards the *B. subtilis* colony that facilitated *S. indica* hyphal growth. On medium containing thiamine, *S. indica* growth speed slowed down when approaching *B. subtilis* colony. There was still no clear explanation of this phenomenon. Since only runner hyphae but no BAS formed close to *B. subtilis* colony, this avoidance might also relate to nutrient availability, in addition to pH (as described in B.3.2). Moreover, the excessive spatial-temporal heterogeneity of soil can cause various microbial population types and community interactions patterns (Minz *et al.*, 2013). By analysing the real-time *S. indica-B. subtilis* synthetic community development at certain spatial set up (such as experiments in this chapter), would provide insights to the understanding on the formation and ecological succession of microbial communities.

To quantify *S. indica* growth with image analysis requires good quality images. Keeping *S. indica* in one plane helps to getting most of the growth details (such as germination, elongation and branching) under the microscope. The growth of *S. indica* on agar medium is 3-dimensional, as the hyphae can grow into the agar and towards the air. This makes it difficult to keep the colony to one focus plane for acquiring all details of *S. indica* growth. Moreover, a sample of *S. indica* growing on agar plate is thick, making it impossible to observe under high magnification (such as 400 times). Therefore, the microfluidic system built in this study was very helpful in obtaining *S. indica* growth at a single cell resolution. This system also provided possibilities for future developments, such as testing different medium and medium

with stain or root exudates on live *S. indica* cell, and analysing *B. subtilis* and *S. indica* interaction under high magnification. This system could also help in introducing live *A. thaliana* root as reported in another study (Massalha *et al.*, 2017) to test the synthetic microbial community behaviour.

Particles forming in *S. indica* hyphae has never been reported before. The particles formation was possibly the constriction of the cytoplasm when it was initiating or proceeding cell division of the hypha. Such processes could also contribute to the beads-like *S. indica* hyphae morphology reported in another study (Singhal *et al.*, 2017b). More explorations are required to understand the germination process.

Understanding *S. indica* hyphae branching is also important, as it could relate to fungal growth stratagem or decision making as shown in other studies (Hanson *et al.*, 2006; Reid *et al.*, 2015). Trials have been made to record the real-time elongation and branching of the hypha with the microfluidic system. Since there was no special structure in the chamber to fix *S. indica* spore, when hyphae expanded, spores were dragged along and moved away from their original position. Under higher magnification (400 times), it was impossible to trace the original recording ones as a slight movement would result in a considerable distance of view field. It was difficult to track several view fields to trace the spore and hyphae due to equipment flaws. Therefore, the recording was done under lower magnification (40 times) for a broader view field (Video 6). However, because the diameter of hyphae is usually 5 μm, the height of chamber was not of ideal dimensions to keep hyphae growth at one layer (Figure 4-14). New designs of chambers are needed for a clearer view of the *S. indica* growth.

With the established observation methods on agar plate, it is possible to do real-time recording and growth quantification of *S. indica* and *B. subtilis* at the microscopic level. Such method can be used for analysing community consists of other microbes or systems containing more than two cultivable species that can be cultivated on plates. The current microfluidic system can be used to record the germination and growth of *S. indica* and other filamentous microbes with similar size. Quantifiable data generated from both the agar plate observation and microfluidic system provide useful information in understanding the growth dynamics of microbes and can be further used in modelling their behaviours. The quantification of microbe growth is not limited to the integrated image intensity. Fluorescent dyes such as pHrodor Red, SYTOX Blue and SYBR Green I can be used in combination with the agar plate observation or microfluidic system for real-time targeted quantifications like microbe intracellular pH level, live and dead cell ratio and distribution.

Chapter 5

Conclusion and future prospects

This Chapter discusses the principal findings of this study in the context of the main objectives outlined in section 1.7, and the directions for further investigation based on this study.

5.1 *S. indica* thiamine auxotrophy

The aim of constructing a defined environment for *S. indica* (**Aim 1**) was sought in **Chapter 2**, by designing and testing the synthetic medium. This synthetic medium not only made possible of *S. indica* cultivating with all known elements, but also allowed for changing important parameters such as thiamine conditions or nitrogen sources to explore *S. indica* growth under different treatments.

Along with the construction of the synthetic medium was the discovery of *S. indica* thiamine auxotrophy. This study is the first to report *S. indica* inability to synthesise thiamine. This discovery was further investigated with genetic analysis of *S. indica* genome and phylogenetic analysis across the fungal kingdom, to understand the evolutionary nature of lacking thiamine biosynthesis genes. *S. indica* and its close relative *S. vermifera* lost thiamine biosynthesis pathway and can only acquire environmental thiamine as the source for their central metabolism. This thiamine auxotrophy is not very wide-spread and does not show any class or sub-class specific trends.

S. indica is an important plant beneficial microbe and a model organism for studying symbiotic interactions between plant roots and fungi (Lahrmann *et al.*, 2013; Weiss *et al.*, 2016; Strehmel *et al.*, 2016). Understanding the *S. indica* nutritional requirements would have a profound influence on biotechnological applications such as its mass production as biofertilizer (Singhal *et al.*, 2017a) or its usage as soil treatment (Rabiey *et al.*, 2017). Future metabolic and physiological studies of *S.*

indica will be enabled by the defined media conditions and identified thiamine auxotrophy in this study.

5.2 Synthetic microbial community of *S. indica* and *B. subtilis*

The construction of minimal microbial community with one fungus and one bacterium (**Aim 2**) was achieved in **Chapter 3**, with the plant beneficial microbes *S. indica* and *B. subtilis*. This is the first study on these two ecologically and functionally relevant species being put together in a defined environment. The presented findings have implications for the engineering and application of minimal synthetic microbial community that aim to establish plant-supporting soil communities.

The success of auxotrophic interactions relating to spatiotemporal effects suggests that consideration should be given to inoculation timing when designing or applying biofertilizers or biocontrol agents to the soil. Indeed, microbial interactions and synergisms are suggested to be crucial for soil fertility, bioproductivity, and ecosystem functioning (Perotto and Bonfante, 1997; Pérez-Jaramillo *et al.*, 2016; Bulgarelli *et al.*, 2013). Plants significantly benefit from symbioses with soil microbes, with benefits ranging from nutrient supply and growth promotion to elevating plant stress resistance (Castillo *et al.*, 2013; Davison, 1988; Kohler *et al.*, 2015; Vessey, 2003; Yurgel *et al.*, 2014). At the same time, soil microbes can interact among themselves or alter each other's interactions with the plants (Lareen *et al.*, 2016; Veresoglou *et al.*, 2012; Fitter and Garbaye, 1994; Kohlmeier *et al.*, 2005). The biochemical basis of these potential multi-level interactions in the soil has remained mostly elusive to date, with few documented cases of amino acid auxotrophies in specific soil bacteria and vitamin provision from plants relating to their root

colonisation (Streit *et al.*, 1996; diCenzo *et al.*, 2015; Nagae *et al.*, 2016). The presented synthetic community of *S. indica* and *B. subtilis* shows that metabolic auxotrophy can directly underpin microbial interactions and growth, and that the success of interactions can be determined by the spatiotemporal organization in the system. This synthetic system allows controlled investigations (and potential optimization) of fungal–bacteria interactions and can be further extended with additional microbes or plants. The resulting minimalist synthetic ecosystem can provide a platform to analyse and control cross-kingdom relationships between plants and their growth-promoting fungi and bacteria (Pieterse *et al.*, 2016), and can be used to understand how plant beneficial microbes interact with each other and enable new applications for precision agriculture such seed pre-treatment and biofertilizer in the future.

5.3 Real-time microscopy analysis and microfluidic system

Morphological study of *S. indica* growth at the microscopic level (**Aim 3**) was performed in **Chapter 4**, with the real-time observation of *S. indica* and *S. indica-B. subtilis* growth, and the construction of microfluidic system. The real-time record of *S. indica* growth in axenic culture and co-culture with *B. subtilis* is the first report on *S. indica* growth at a scale of tens of minutes. The constructed microfluidic system provides a framework for future studies of *S. indica* at single cell resolution.

The real-time growth of *S. indica* in its axenic culture and co-culture with *B. subtilis* helps to elucidate the development of *S. indica* colony. Different hyphae structures were observed under different growth treatment (with or without thiamine or *B. subtilis*). The structures relating to different nutrient status is in general agreement with previous morphological studies on other similar fungi (Bago *et al.*,

1998; Bago *et al.*, 2004). The time-lapse microscopy could contribute to the understanding of interactions between *S. indica* and *B. subtilis* and extend the interaction to a quantifiable level.

Emerging studies have employed the microfluidics to create a controllable environment for analysing fungi (Grünberger *et al.*, 2016), bacteria (Prindle *et al.*, 2015) and plants (Massalha *et al.*, 2017). Inspired by those, this study constructed microfluidic system that helped to examine *S. indica* growth at a single-cell level. The observed *S. indica* germination pattern has never been reported before. The constructed microfluidic system represents a new approach of observing *S. indica* growth and can be expanded to analysing *S. indica* interaction with *B. subtilis* in future.

5.4 Reductionist approach of studying microbial community

Synthetic community engineering is essentially a reductionist approach, which studies a simple microbial community to understand the native community it resembles (Little *et al.*, 2008; De Roy *et al.*, 2014). To accurately represent the natural microbial community with synthetic one is challenging, because of the complexity of natural microbial community and the environmental factors that can influence it (Johns *et al.*, 2016; Widder *et al.*, 2016). An incomplete understanding of ruling principles that determine microbial community structure and dynamics could lead to a biased prediction (Brenner *et al.*, 2008; Weiher *et al.*, 2011).

In this study, the synthetic microbial community consisted of one fungal and one bacterial species, representing microbes from two different kingdoms. These two organisms are functionally and ecologically relevant, as both are plant beneficial microbes existing in the soil. This relevance can help in effectively associating them

together to develop interactions. Both organisms are relatively well-studied as model organisms for their growth and function, and therefore the risk of incomplete understanding of these organisms is reduced. The well-defined synthetic medium ruled out many unknown factors that might affect their interaction. Thus, this synthetic community is practical for the analysis on organisms' interaction under a controlled environment. Moreover, this is a minimal community consisting of two organisms. It serves as a basics module of microbial interactions and can be enriched with more organisms for developing systems with higher complexity such as a microbial community with more than two organisms or a microbe-plant community. Moreover, the plant beneficial traits of these two organisms could help in pushing such system towards the application of generating plant beneficial effects.

Still, there are limitations of using this synthetic microbial community to understand the natural microbial community. Since these interactions were observed in the absence of other organisms, they might not be the same as observed in this study when the system becomes more complex (such as with additional plants or microbes). The established relationship in this system might be interfered, such as the thiamine provision from *B. subtilis* to *S. indica* might switches to another organism to *S. indica*. It is also possible that these interactions between *S. indica* and *B. subtilis* become neglectable when the additional organism have more influential power on *S. indica* and *B. subtilis* growth. The overall interactions could change when the system is placed in another environment (different medium, temperature or solid/liquid phase), such as described in appendix B, where *S. indica* and *B. subtilis* were co-cultured on medium containing thiamine, no auxotrophic interaction was observed and different interaction patterns were developed. Since the natural environment are not always constant, the *S. indica- B. subtilis* system developed in this study cannot

predict how these microbes can interact in various environment. Therefore, other approaches could be applied to compensate these limitations from this reductionist method, such as the omics analysis on genetics and metabolomics to understand these microbial interactions in new environment, or computational modelling on each organism growth in a system to predict possible interactions. Further investigation is required to analyse the developed fungal-bacterial system and expand its capacity with higher complexity, so that to gain a more comprehensive understanding of the microbial community and its potential applications. These possible directions will be discussed in the following section (5.5).

5.5 Prospects for future research

The synthetic medium developed in this study (section 2.3.1) is not yet a minimal medium for *S. indica* growth. The salts and microelements in the recipe have not been tested for the individual effect on *S. indica* growth. Development of this synthetic medium towards a minimal medium will provide a further understanding of *S. indica* nutritional requirement and will help in the designing of medium environment for *S. indica* interactions with other organisms.

A complete or partial loss of thiamine biosynthesis genes can be observed in different fungi from different classes, rather than being confined to a specific class (section 2.4.5). This loss might relate to the ecology and lifestyle of these species, and would require further analyses to understand the correlations.

As indicated in appendix B, the interaction patterns between *S. indica* and *B. subtilis* (colonies overlapping or avoidance) is adjustable by controlling environmental parameters such as thiamine condition, nitrogen source or medium pH. Such changes would provide insights into the formation and dynamics of microbial

communities. The fundamental causes of such switch on interaction patterns remain to be explored. Metabolic profiling of extracellular metabolites and their diffusion could be potential directions towards a complete understanding. Also, further metabolic analysis of the organic acids from cross-feeding experiments is needed, because there are still many unknown peaks from IC measurement (representing unidentified metabolites) that changed drastically during the cross-feeding.

Thiamine analysis is an unsolved question across this study. While findings described in section 3.4.1 strongly suggest a *B. subtilis*-linked thiamine provision which then promotes *S. indica* growth, the attempts to quantify thiamine from agar plate co-cultures has failed. This failure could occur presumably due to a combination of thiamine consumption and sensitivity limitations of available thiamine assays (Lu and Frank, 2008; Edwards *et al.*, 2017). The working concentration of thiamine in the synthetic medium was 150 µg/l. This concentration of thiamine supported a well propagated *S. indica* culture with dense hyphae layers and continual spore production. Even at 1.5 µg/l thiamine, a concentration already outside of the established detection limit using thiochrome assay and a plate reader, *S. indica* was still forming hyphae and generating spores (Figure 2-8). The thiamine provided by *B. subtilis*, which only maintained a thin layer of *S. indica* hypha, could not be measured using current resources. In future, it is advised to seek available instruments capable of doing such analysis, such as a HPLC machine with a fluorescence detector. Knowing how much thiamine *B. subtilis* provided to *S. indica* in the co-culture on the solid medium would provide insights into a further understanding of this auxotrophy interaction. Measuring thiamine storage in *S. indica* spore that allows for germination and initial hyphae expansion would help in deducing its thiamine requirement for reproduction.

Analysing thiamine in *A. thaliana* root exudates would be useful to understand the plant-fungi interaction.

Trials have been made in appendix C to expand the synthetic microbial community of *S. indica* and *B. subtilis* with the inclusion of *A. thaliana*, to explore the potential plant beneficial effect of this fungal-bacterial community. A full understanding of microbial interactions even in a 2-species case requires the consideration of biophysical factors and metabolic outputs at microscales, making these studies very challenging. This study made the first step in this direction for a plant/soil relevant system. Future work is needed to create an appropriate environment for cultivating the three species (*A. thaliana*, *S. indica* and *B. subtilis*) together. The microfluidic system would be the next stage of putting those three organisms together, as described in another study that root-bacteria interaction can be analysed in real-time (Massalha *et al.*, 2017).

Based on the detailed observation on *S. indica* growth (section 4.4.3), the microfluidic system can be further enhanced. Different chip designs which allowing the fixation of *S. indica* spores could contribute to a better observation on hyphae growth. A microfluidic chamber of lower height will be under investigation for such purpose.

The quantification method of microbe growth can be enriched with fluorescence probes such as pHrodor Red, SYTOX Blue and SYBR Green I. These fluorescence probes could provide information on microbe intracellular pH level, live and dead cell ratio and distribution. By combining the fluorescence probes with the established time-lapse microscopy, it is possible to reveal the dynamics of these features in microbes, which might contribute to a better understanding of the microbe growth.

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Appendix A

Categories	Species	KEGG pathways				
		K14154;(THI6)[112]	K00877;(THI20)[105]	K18278;(THI5)[78]	K03146;(THI4)[108]	K00949;(thiN, THI80)[108]
Ascomycota -KEGG	Saccharomyces cerevisiae	YPL214C	YOL055C;YPL258C	YNL332W;YJR156C; YDL244W;YFL058W	YGR144W	YOR143C
	Ashbya gossypii	AGOS_AFL110C	no	AGOS_AER451W	AGOS_AAL137W	AGOS_AFR387C
	Eremothecium cymbalariae	Ecym_2185	no	Ecym_3622	Ecym_6012	Ecym_4555
	Kluyveromyces lactis	KLLA0B02134g	KLLA0B13926g	KLLA0C19085g	KLLA0A00198g	KLLA0B03784g;KLLA0E03257g
	Lachancea thermotolerans	KLTH0H03784g	KLTH0F06732g	KLTH0B02574g	KLTH0B03916g	KLTH0G02508g
	Vanderwaltozyma polyspora	Kpol_1002p43	Kpol_1045p51	no	Kpol_1050p31	Kpol_543p11
	Zygosaccharomyces rouxii	ZYRO0E07216g	ZYRO0A05280g	ZYRO0A11000g	ZYRO0G11726g	ZYRO0D11374g
	Naumovozyma castellii	NCAS_0A01010	NCAS_0C03670	no	NCAS_0D01750	NCAS_0H02310
	Naumovozyma dairenensis	NDAI_0F01540	NDAI_0G03010	no	NDAI_0C02860	NDAI_0C01350
	Tetrapisispora phaffii	TPHA_0J02470	TPHA_0P00590	no	TPHA_0A04790	TPHA_0J02800
	Tetrapisispora blattae	TBLA_0A06110	TBLA_0G00850; TBLA_0B04160; TBLA_0B04170	no	TBLA_0H00760;TBLA_0B09390	TBLA_0D01920
	Torulaspora delbrueckii	TDEL_0E05400	TDEL_0D04490	TDEL_0E03920	TDEL_0H00200	TDEL_0A03430
	Kazachstania africana	KAFR_0K00230	KAFR_0C00950	no	no	KAFR_0E02510
	Komagataella phaffii	PAS_chr3_0843	PAS_chr3_0842	PAS_chr4_0065	PAS_chr3_0648	PAS_chr3_0052
	Debaryomyces hansenii	DEHA2G01166g	DEHA2E20878g	DEHA2A02420g	DEHA2F16654g	DEHA2G07788g

	Scheffersomyces stipitis	PICST_77822	PICST_70077	PICST_30906	PICST_50942	PICST_59866;PICST_46967
	Meyerozyma guilliermondii	PGUG_04644	PGUG_05036	PGUG_02619	PGUG_04211	PGUG_02670
	Spathaspora passalidarum	SPAPADRAFT_1409	SPAPADRAFT_6082	no	SPAPADRAFT_5139	SPAPADRAFT_6130
	Lodderomyces elongisporus	LELG_02003	LELG_01321	no	LELG_02871	LELG_03867
	Candida albicans	CAALFM_C302860W	CAALFM_C203370W	CAALFM_CR09290W	CAALFM_C305130C	CAALFM_C503840W
	Candida tropicalis	CTRG_02560	CTRG_01185	CTRG_05917	CTRG_02525	CTRG_03190
	Candida orthopsilosis	CORT_0B04690	CORT_0A11240	CORT_0G01430	CORT_0C04770	CORT_0E05180
	Candida dubliniensis	CD36_82850	CD36_18120	CD36_34630	CD36_85100	CD36_53570
	Yarrowia lipolytica	YALI0C15554g	YALI0E04224g	no	YALI0A09768g	YALI0E21351g
	Clavispora lusitaniae ATCC 42720	CLUG_03522	CLUG_02764	no	CLUG_04547	CLUG_03618
	Neurospora crassa	NCU11173	NCU07849	NCU09345	NCU06110	NCU04944
	Sordaria macrospora	SMAC_02265	SMAC_02123	SMAC_07852	SMAC_06409	SMAC_05633
	Podospora anserina	PODANSg4855	PODANSg5541	no	PODANSg1687	PODANSg270
	Thielavia terrestris	THITE_2119543	THITE_2120167	THITE_2117524	THITE_2117402	THITE_65995
	Chaetomium thermophilum	CTHT_0024040	no	no	CTHT_0017160	no
	Magnaporthe oryzae	MGG_06448	MGG_16900	no	MGG_03098	MGG_09347
	Phaeoacremonium minimum	UCRPA7_1662	UCRPA7_3860	UCRPA7_4536	UCRPA7_2510	UCRPA7_4064
	Fusarium graminearum	FGSG_07264	FGSG_04128	FGSG_01672	FGSG_02469	FGSG_02454
	Fusarium verticillioides	FVEG_05316	FVEG_10314	FVEG_09760	FVEG_09077	FVEG_09060
	Fusarium oxysporum	FOXG_02153	FOXG_11509	FOXG_11035	FOXG_10428	FOXG_10411
	Nectria haematococca	NECHADRAFT_6861	NECHADRAFT_9448	NECHADRAFT_6510	NECHADRAFT_6189	NECHADRAFT_6190

	Trichoderma reesei	TRIREDRAFT_7599	TRIREDRAFT_5906	TRIREDRAFT_1216	TRIREDRAFT_6860	TRIREDRAFT_5861
	Metarhizium acridum	MAC_00604	no	MAC_01565	MAC_01186	MAC_07368
	Metarhizium robertsii	MAA_05935	MAA_02490	MAA_02402	MAA_05549	MAA_05519
	Cordyceps militaris	CCM_01506	CCM_09434	CCM_05035	CCM_01639	CCM_02875
	Purpureocillium lilacinum	no	VFPFJ_02365	VFPFJ_02501	VFPFJ_09336	VFPFJ_09294
	Verticillium alfalfae	no	VDBG_06636	VDBG_09061	VDBG_03908	VDBG_08599
	Verticillium dahliae	VDAG_06345	VDAG_05690	VDAG_07211	VDAG_01137	VDAG_08269
	Colletotrichum fioriniae	CFIO01_10597	CFIO01_08677	CFIO01_07592	CFIO01_04759	CFIO01_06008
	Eutypa lata	UCREL1_11567	UCREL1_179	UCREL1_1445	UCREL1_6105	UCREL1_2791
	Sclerotinia sclerotiorum	SS1G_13563	SS1G_00072	SS1G_10697	SS1G_09727	SS1G_03631
	Botrytis cinerea	BC1G_10430	no	BC1G_04245	BC1G_11634	BC1G_03577
	Marssonina brunnea	MBM_00376	MBM_07084	no	MBM_01288	MBM_03290
	Glarea lozoyensis	GLAREA_11575	GLAREA_06915	GLAREA_01701	GLAREA_02091	GLAREA_08241
	Aspergillus nidulans	AN3878.2	no	AN8009.2	AN3928.2	AN9488.2
	Aspergillus fumigatus	AFUA_2G08970	AFUA_2G10740	AFUA_5G02470	AFUA_6G08360	AFUA_7G05410
	Aspergillus oryzae	AOR_1_1340054	AOR_1_948054	AOR_1_622134	AOR_1_156154	AOR_1_3020174
	Aspergillus niger	ANI_1_588104	ANI_1_2342024	ANI_1_1450024	ANI_1_228094	no
	Aspergillus flavus	AFLA_046020	AFLA_043830	AFLA_004650	AFLA_037160	no
	Aspergillus clavatus	ACLA_080810	ACLA_069750	ACLA_002690	ACLA_082310	ACLA_007100
	Aspergillus fischeri	NFIA_084530	NFIA_086080	NFIA_039610	NFIA_054010	NFIA_026620
	Penicillium rubens	Pc13g03600	Pc22g21020	Pc21g15700	Pc22g07960	Pc12g06110

	Penicillium digitatum	PDIP_84220	PDIP_63900	PDIP_48680	PDIP_32520	PDIP_54580
	Coccidioides immitis	CIMG_00798	CIMG_07892	CIMG_09722	CIMG_01115	CIMG_05245
	Coccidioides posadasii	CPC735_055050	CPC735_041310	CPC735_023240	CPC735_052570	CPC735_065040
	Paracoccidioides lutzii Pb01	PAAG_02219	PAAG_06485	PAAG_06107	PAAG_01125;PAAG_03817	PAAG_00181
	Paracoccidioides brasiliensis Pb18	PADG_05357	no	PADG_08010	PADG_03708	PADG_12236
	Uncinocarpus reesii	UREG_00763	no	UREG_04656	UREG_01058	UREG_06383
	Trichophyton benhamiae	ARB_04892	ARB_05224	ARB_06596	ARB_07940	ARB_04755
	Trichophyton verrucosum	TRV_07077	TRV_02263	TRV_04807	TRV_04098	TRV_03452
	Histoplasma capsulatum	HCAG_07304	HCAG_08708	HCAG_03261	HCAG_05915	HCAG_03837
	Parastagonospora nodorum	SNOG_13715	no	SNOG_11769	SNOG_05965	SNOG_05935
	Pyrenophora teres	PTT_18545	PTT_11285	PTT_08439	PTT_08482	PTT_07110
	Bipolaris zeicola	COCCADRAFT_6682	no	COCCADRAFT_3803	COCCADRAFT_2702	COCCADRAFT_1075
	Bipolaris sorokiniana	COCSADRAFT_3272	no	COCSADRAFT_2452	COCSADRAFT_3826	COCSADRAFT_1211
	Bipolaris oryzae	COCMIDRAFT_2997	no	COCMIDRAFT_1091	COCMIDRAFT_3003	COCMIDRAFT_1042
	Zymoseptoria tritici	MYCGRDRAFT_6986	MYCGRDRAFT_6787	MYCGRDRAFT_1022	MYCGRDRAFT_4426	MYCGRDRAFT_1740
	Pseudocercospora fijiensis	MYCFIDRAFT_1523	MYCFIDRAFT_4382	MYCFIDRAFT_2033	MYCFIDRAFT_2118	MYCFIDRAFT_2305
	Baudoinia panamericana	BAUCODRAFT_3160	BAUCODRAFT_5688	BAUCODRAFT_7653	BAUCODRAFT_6266	BAUCODRAFT_1223
	Neofusicoccum parvum	UCRNP2_1656	UCRNP2_4157	UCRNP2_8417	UCRNP2_7340	UCRNP2_9478
	Tuber melanosporum	GSTUM_000017100	GSTUM_000017090	no	no	GSTUM_000017320
	Schizosaccharomyces pombe	SPAC23H4.10c	SPBP8B7.18c; SPBP8B7.17c;	SPCC1223.02	SPBC26H8.01	no

			SPCC18B5.05c			
Basidiomycota-KEGG	Cryptococcus neoformans var. neoformans B-3501A	CNBH3540	CNBG4610	no	no	CNBH1110
	Cryptococcus gattii	CGB_H4330W	CGB_G6540C	no	no	CGB_H1700C
	Tremella mesenterica	no	no	no	no	TREMEDRAFT_2504
	Wallemia mellicola	WALSEDRAFT_3524	WALSEDRAFT_5975	WALSEDRAFT_5273	WALSEDRAFT_3218	WALSEDRAFT_6013
	Wallemia ichthyophaga	J056_004019	J056_000079	J056_002573	J056_001533	no
	Ustilago maydis	UMAG_02614	UMAG_03618	UMAG_00816	UMAG_11400	no
	Anthracocystis flocculosa	PFL1_01880	PFL1_02445	PFL1_06084	PFL1_06351	no
	Malassezia globosa	MGL_0253	MGL_1338	no	MGL_1314	MGL_2385
	Melampsora larici-populina	MELLADRAFT_6371	MELLADRAFT_2493	MELLADRAFT_6701	MELLADRAFT_5383	MELLADRAFT_9210
Agaricomycetes-KEGG	Postia placenta	POSPLDRAFT_8852	no	no	no	POSPLDRAFT_3618
	Trametes versicolor	TRAVEDRAFT_2652	TRAVEDRAFT_1467	no	TRAVEDRAFT_7151	TRAVEDRAFT_1689
	Dichomitus squalens	DICSQDRAFT_7904	DICSQDRAFT_7174	no	DICSQDRAFT_1348	DICSQDRAFT_5203
	Phanerochaete carnosa	PHACADRAFT_1232	PHACADRAFT_2095	no	PHACADRAFT_1772	PHACADRAFT_1399
	Stereum hirsutum	STEHIDRAFT_1711	STEHIDRAFT_1713	no	STEHIDRAFT_1116	STEHIDRAFT_4981
	Heterobasidion irregulare	HETIRDRAFT_4525	HETIRDRAFT_4701	no	HETIRDRAFT_4400	HETIRDRAFT_1634
	Punctularia strigosozonata	PUNSTDRAFT_1420	PUNSTDRAFT_1444	no	PUNSTDRAFT_4241	PUNSTDRAFT_7760
	Fomitiporia mediterranea	FOMMEDRAFT_1341	FOMMEDRAFT_1047	no	FOMMEDRAFT_2369	FOMMEDRAFT_1021
	Gloeophyllum trabeum	GLOTRDRAFT_1153	GLOTRDRAFT_8211	GLOTRDRAFT_1071	GLOTRDRAFT_7721	GLOTRDRAFT_8123
	Moniliophthora perniciosa	no	no	no	no	MPER_15598
	Moniliophthora roreri	Moror_17729	Moror_11019	no	Moror_11852	Moror_13116

	Coprinopsis cinerea	CC1G_03317	CC1G_01447	no	CC1G_04976	CC1G_06388
	Schizophyllum commune	SCHCODRAFT_6898	SCHCODRAFT_7839	no	SCHCODRAFT_8482	SCHCODRAFT_6161
	Agaricus bisporus var. bisporus H97	AGABI2DRAFT2296	AGABI2DRAFT1836	no	AGABI2DRAFT7278	AGABI2DRAFT2015
	Coniophora puteana	CONPUDRAFT_1361	CONPUDRAFT_1185	no	CONPUDRAFT_1042	no
	Serpula lacrymans	SERLADRAFT_3481	SERLADRAFT_4519	no	SERLADRAFT_4701	SERLADRAFT_3560
Agaricomycetes-BLAST	Serendipita indica	no	no	no	15% 2e-06 48% CCA73069.1	84%1e-2632%CCA69955.1
	Serendipita vermifera	no	no	no	12% 1e-05 KIM29805.1	KIM25589.1
	Heterobasidion annosum 03012	29% 6e-28 4 AOSL01000330.1	35% 6e-25 6 AOSL01000390.1	no	no	32% 7e-10 4 AOSL01000004.1
	Daedalea quercina L-15889	37% 2e-28 6 KV429048.1	34% 5e-20 4 KV429034.1	no	80% 5e-79 6 KV429035.1	45% 8e-16 3 KV429038.1
	Grifola frondosa	36% 4e-27 4 LUGG01000022.1	42% 5e-11 4 LUGG01000004.1	no	76% 5e-79 7 LUGG01000011.1	48% 3e-14 4 LUGG01000009.1
	Rhizoctonia solani AG-1 IA	44% 5e-19 4 MRJL02000155.1	47% 8e-23 6 MRJL02000100.1	91% 2e-85 5 MRJL02000032.1	60% 2e-50 6 MRJL02000155.1	no
	Laetiporus sulphureus	42% 3e-22 2 KV427608.1	43% 2e-20 4 KV427607.1	no	77% 7e-82 7 KV427612.1	31% 3e-09 4 KV427618.1
	Gelatoporia subvermispora B	40% 1e-26 4 KB445796.1	29% 8e-15 5 KB445803.1	no	79% 6e-86 6 KB445793.1	46% 9e-11 3 KB445804.1
	Polyporus brumalis	48% 3e-32 6 MARA01000010.1	37% 1e-17 5 MARA01000012.1	no	82% 4e-88 6 MARA01000001.1	40% 5e-13 2 MARA01000001.1
	Fomitopsis palustris	37% 6e-25 5 MJIM01000021.1	35% 7e-22 4 MJIM01000005.1	no	75% 5e-78 6 MJIM01000006.1	38% 2e-10 3 MJIM01000018.1
	Schizopora paradoxa	29% 2e-25 3 KQ085895.1	44% 2e-19 6 KQ085885.1	no	80% 4e-85 5 KQ085992.1	24% 9e-07 3 KQ085959.1
	Irpex lacteus	45% 2e-26 5 MQVO02000005.1	55% 3e-20 3 MQVO02000002.1	no	87% 2e-84 6 MQVO02000001.1	no
	Exidia glandulosa HHB12029	no	no	no	72% 6e-70 8 KV426150.1	37% 3e-13 4 KV426213.1

	Limonomyces culmigenus	31% 3e-26 5 LCTX01000034.1	36% 1e-23 6 LCTX01008542.1	no	80% 6e-71 7 LCTX01000944.1	37% 2e-11 5 LCTX01000366.1
	Leucoagaricus gongylophorus Ac12	41% 8e-36 7 ANIS01001210.1	32% 2e-20 5 ANIS01003499.1	no	no	45% 9e-14 4 ANIS01009346.1
	Plicaturopsis crispa FD-325 SS-3	35% 1e-21 4 KN832556.1	18% 2e-14 2 KN832587.1	no	85% 1e-58 5 KN832558.1	no
	Pleurotus salmoneostramineus	37% 3e-27 6 BEWF01000131.1	35% 2e-23 5 BEWF01000671.1	no	no	32% 9e-09 4 BEWF01000083.1
	Omphalotus olearius	1e-29 5 AHIW01000015.1	33% 2e-23 5 AHIW01000369.1	no	84% 7e-82 6 JH814821.1	28% 3e-08 4 AHIW01000593.1
	Phlebopus portentosus	54% 3e-35 7 KN880369.1	44% 2e-10 6 KN880381.1	no	86% 1e-56 4 KN880364.1	45% 2e-08 4 KN880383.1
	Lignosus rhinocerotis	44% 3e-35 6 KN049986.1	no	no	84% 5e-85 5 KN050003.1	40% 2e-15 4 KN050056.1
	Sparassis latifolia	42% 2e-22 4 LWKX01000001.1	37% 9e-17 5 LWKX01000024.1	no	78% 6e-82 7 LWKX01000054.1	40% 7e-10 3 LWKX01000042.1
	Flammulina velutipes	40% 1e-30 5 BDAN01001302.1	29% 6e-19 4 BDAN01001772.1	no	79% 3e-85 6 BDAN01003851.1	41% 3e-08 4 BDAN01002856.1
	Hebeloma cylindrosporum h7	40% 5e-22 5 KN831772.1	33% 4e-13 4 KN831768.1	no	75% 5e-78 7 KN831776.1	41% 8e-09 3 KN831784.1
	Paxillus involutus ATCC 200175	44% 3e-32 5 KN819327.1	38% 7e-17 6 KN819362.1	no	82% 3e-83 6 KN819330.1	30% 1e-10 4 KN820324.1
	Volvariella volvacea V23	45% 8e-26 4 KB722737.1	40% 4e-22 6 KB722758.1	no	79% 8e-82 7 KB722736.1	25% 5e-07 2 KB722743.1
	Trametes hirsuta	69% 7e-29 5 CP019378.1	40% 2e-17 3 CP019376.1	no	84% 5e-69 4 CP019377.1	40% 7e-13 2 CP019380.1
	Fistulina hepatica ATCC64428	35% 4e-22 5 KN882110.1	32% 1e-17 5 KN882020.1	no	71% 4e-77 6 KN882097.1	30% 3e-08 3 KN881671.1
	Sphaerobolus stellatus SS14	no	41% 6e-20 3 KN837227.1	no	79% 6e-83 6 KN837502.1	no
	Laccaria amethystina LaAM-08-1	32% 1e-23 4 KN838537.1	28% 6e-21 4 KN838766.1	no	no	37% 8e-11 2 KN838536.1
	Phlebiopsis gigantea 11061_1 CR5-6	39% 3e-37 5 KN840514.1	39% 2e-19 6 KN840496.1	no	81% 1e-85 6 KN840523.1	37% 8e-13 5 KN840555.1

	Fomitopsis pinicola FP-58527	33% 2e-25 5 KE504153.1	35% 2e-19 6 KE504166.1	no	81% 8e-81 7 KE504124.1	36% 0.024 3 KE504123.1
	Amanita bisporigera	no	22% 5e-13 4 MIPV01008695.1	no	53% 5e-46 6 MIPV01006385.1	31% 3e-10 4 MIPV01003511.1
	Hypholoma sublateritium FD-334 SS-4	38% 5e-26 6 KN817523.1	39% 3e-26 5 KN817581.1	no	81% 3e-80 7 KN817560.1	45% 3e-13 4 KN817546.1
	Amanita jacksonii TRTC168611	no	40% 4e-18 4 KI547035.1	no	86% 3e-81 5 KI546600.1	no
	Phellinus noxius OVT-YTM/97	29% 7e-18 4 AYOR01000087.1	39% 4e-18 5 AYOR01000052.1	no	79% 4e-78 6 AYOR01000401.1	42% 1e-10 4 AYOR01000046.1
	Auricularia auricula-judae	25% 1e-20 3 NCVV01000002.1	46% 6e-24 7 NCVV01000025.1	no	77% 1e-66 8 NCVV01000006.1	45% 5e-11 4 NCVV01000032.1
	Ganoderma lucidum BCRC 37177	47% 5e-32 6 BACH01001325.1	40% 8e-22 6 BACH01000129.1	no	82% 3e-86 4 BACH01001218.1	40% 3e-16 4 BACH01002525.1
	Neolentinus lepideus HHB14362 ss-1	46% 1e-29 6 KV425630.1	38% 1e-20 6 KV425660.1	97% 3e-98 7 KV425552.1	86% 2e-84 5 KV425585.1	38% 5e-11 3 KV425571.1
	Pisolithus tinctorius Marx 270	42% 3e-29 5 KN831953.1	41% 6e-20 6 KN831966.1	no	80% 9e-86 6 KN831980.1	43% 3e-15 4 KN831974.1
	Taiwanofungus camphoratus	36% 3e-23 4 JNBV01000009.1	45% 3e-18 4 JNBV01000002.1	no	79% 1e-83 7 JNBV01000005.1	41% 5e-09 3 JNBV01000004.1
	Phlebia centrifuga	40% 5e-32 6 MLYV01000832.1	39% 8e-26 6 MLYV01001249.1	no	77% 6e-83 6 MLYV01001281.1	39% 4e-12 5 MLYV01001030.1
	Scleroderma citrinum Foug A	47% 3e-31 6 KN822050.1	26% 2e-11 4 KN822055.1	no	78% 6e-83 6 KN822035.1	43% 7e-09 5 KN822009.1
	Botryobasidium botryosum FD-172 SS1	61% 3e-29 5 KL198016.1	40% 3e-21 6 KL198021.1	no	no	29% 1e-07 4 KL198030.1
	Sanghuangporus baumii	30% 8e-22 3 LNZH02000198.1	54% 3e-30 7 LNZH02000106.1	no	81% 6e-85 6 LNZH02000208.1	38% 2e-07 3 LNZH02000171.1
	Jaapia argillacea MUCL 33604	57% 3e-26 6 KL197719.1	34% 8e-21 4 KL197715.1	no	75% 1e-78 5 KL197711.1	37% 7e-10 3 KL197724.1
	Hypsizygus marmoreus	43% 4e-20 4 NIBX01000021.1	41% 2e-20 6 NIBX01000059.1	no	78% 2e-73 5 NIBX01000158.1	no
	Galerina marginata CBS 339.88	31% 1e-21 5 KL142381.1	56% 2e-15 5 KL142370.1	no	79% 9e-55 4 KL142369.1	32% 6e-05 3 KL142379.1

	Hydnomerulius pinastri MD-312	63% 8e-32 5 KN839838.1	38% 6e-15 6 KN839851.1	no	78% 6e-85 6 KN839854.1	no
	Gymnopus luxurians FD-317 M1	29% 1e-18 2 KN834754.1	23% 3e-15 4 KN834851.1	no	79% 8e-81 7 KN834849.1	36% 2e-04 2 KN834753.1
	Athelia rolfsii	41% 3e-30 5 JZWR02002089.1	41% 1e-23 8 JZWR02002285.1	no	68% 7e-57 7 JZWR02002434.1	48% 2e-14 4 JZWR02001588.1
	Phanerochaete chrysosporium	24% 2e-19 2 MJGA01000005.1	36% 5e-14 4 MJGA01000009.1	no	76% 7e-23 4 MJGA01000006.1	53% 1e-12 3 MJGA01000002.1
	Wolfiporia cocos MD-104	37% 5e-26 5 KB468146.1	39% 2e-17 4 KB467942.1	no	81% 1e-82 7 KB468053.1	40% 8e-11 3 KB468124.1
	Trametes polyzona	51% 3e-34 6 MKKQ01000611.1	38% 2e-26 6 MKKQ01002362.1	no	84% 6e-89 5 MKKQ01005151.1	36% 1e-12 4 MKKQ01004351.1
	Pleurotus ostreatus PC15	47% 5e-24 3 KL198004.1	36% 7e-19 4 KL198007.1	no	82% 1e-85 6 KL198010.1	39% 7e-09 3 KL198011.1
	Amanita brunnescens Koide BX004	42% 3e-30 7 JNHV01016250.1	34% 3e-17 5 JNHV01016870.1	no	79% 2e-80 7 JNHV01011828.1	29% 3e-09 4 JNHV01016842.1
	Lyophyllum decastes	39% 4e-25 5 BCJR01000012.1	33% 6e-17 4 BCJR01000001.1	no	82% 2e-77 7 BCJR01000018.1	32% 6e-07 4 BCJR01000009.1
	Coprinopsis strossmayeri	43% 2e-27 7 FTPT01000096.1	35% 1e-16 5 FTPT01000018.1	no	75% 4e-79 7 FTPT01000071.1	41% 6e-11 3 FTPT01000056.1
	Lentinus polychrous	36% 1e-35 5 JSYW01000324.1	41% 1e-26 6 JSYW01000111.1	no	81% 6e-70 6 JSYW01002102.1	40% 2e-14 5 JSYW01003295.1
	Lentinula edodes	31% 9e-21 2 LSDU01000025.1	30% 1e-14 5 LSDU01000031.1	no	78% 1e-80 7 LSDU01000029.1	no
	Leucoagaricus sp. SymC.cos	40% 5e-21 4 KQ962027.1	25% 2e-09 3 KQ961944.1	no	77% 4e-81 6 KQ962167.1	50% 1e-13 5 KQ961965.1

Appendix B

Different interaction patterns of *S. indica* and *B. subtilis*

B.1 Introduction

The *S. indica* and *B. subtilis* co-culture was analysed on medium without thiamine in chapter 3. For a comprehensive understanding of how this co-culture performs on the synthetic medium, preliminary tests are done using the synthetic medium containing thiamine and different nitrogen sources. Different interaction patterns are found when the co-culture was supplied with particular nitrogen sources and is associated with the environmental pH.

B.2 Materials and Methods

B.2.1 Establishing *S. indica* and *B. subtilis* co-culture

The co-culturing of *S. indica* and *B. subtilis* was initiated on agar medium as indicated in Figure 3-1, the same as described in section 3.3.3. Experiments were carried out on 60 mm petri dishes (Ref: 1007, Corning), filled with 6 ml of agar medium prepared as mentioned in section 2.3.1. Synthetic media with thiamine containing ammonium, glutamine or nitrate as the sole nitrogen source were used to create different environments for the microbial interaction. *S. indica* spore suspension was inoculated 2 µl on the left side of the plates. At approximately 2 cm distance to the right of the inoculum, either a 2 µl "mock" solution (10mM $MgCl_2$) or a 2 µl *B. subtilis* culture were placed. Plates were sealed with parafilm and incubated statically at 30℃ for 2 weeks. Images were taken with a gel doc system (G:Box EF, Syngene).

B.2.2 Bromocresol purple test on the pH of co-culture

Equipments and inoculation schemes were the same as mentioned in section B.2.1. Bromocresol purple was added to liquid medium before sterilisation at the final concentration of 0.02 g/l as mentioned in section 2.3.1. Synthetic media containing ammonium or glutamine as the sole nitrogen source were used. Treatment of glutamine medium at initial pH 5 was prepared by adjusting liquid synthetic medium containing glutamine as the sole nitrogen source to pH 5 with 0.1M HCl before sterilisation. Images were taken after 2 weeks with a commercial camera (8-megapixel camera with *f*/2.2 aperture) for recording colour indication from pH indicator (lab equipment such as gel doc system could not capture coloured images).

B.3 Results

B.3.1 *S. indica* and *B. subtilis* interacted in different patterns on solid media containing thiamine

On the media containing thiamine, two distinct interaction patterns between *S. indica* and *B. subtilis* were observed: *S. indica* hypha merged into *B. subtilis* biofilm, or expanded while avoiding *B. subtilis* colony. While thiamine was present in the synthetic medium and ammonium was the sole nitrogen source, *S. indica* avoided *B. subtilis*, leaving an apparent gap between the two colonies. Instead, when glutamine was the sole nitrogen source, *S. indica* colony expanded and overlapped with *B. subtilis* (Figure B-1).

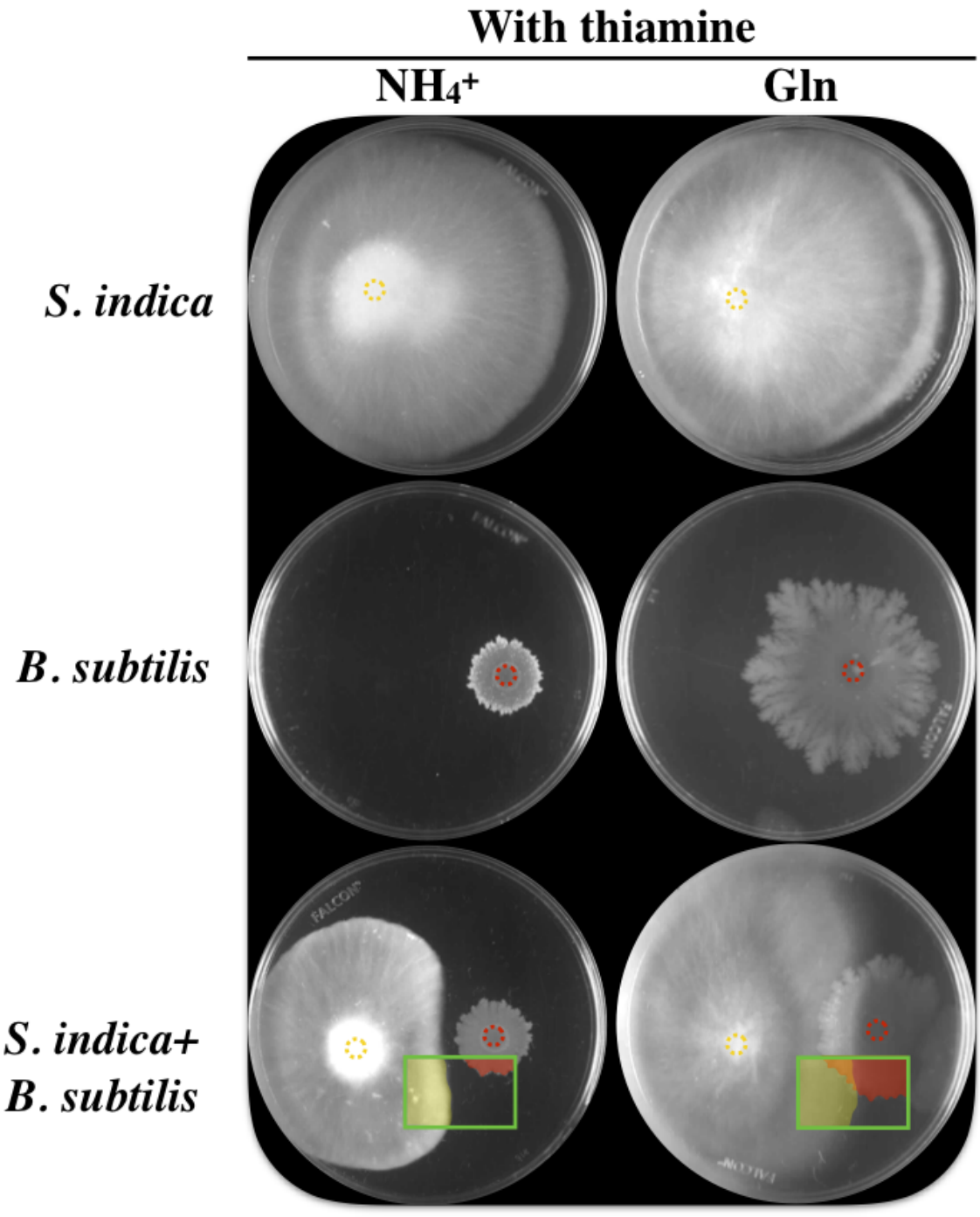


Figure B-1: *S. indica* and *B. subtilis* growth on synthetic media containing thiamine. Rows from top to bottom show growth of monocultures of *S. indica*, *B. subtilis*, and their co-culture, respectively. The left and right columns show growth on medium containing ammonium or glutamine as the sole nitrogen source, respectively. The yellow dotted circle on the images indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis* inoculation point. Green square highlights the colonies with pseudo-colour, where the area covered by *S. indica* hyphae is shown in yellow, and the *B. subtilis* colony is shown in red. The areas were manually drawn with software keynote. When both organisms

were cultured together (bottom row), *S. indica* and *B. subtilis* were inoculated on the left and right of the plate, with a 20 mm space in between. The diameter of each plate is 60 mm. Plates shown are representative of at least three replicates for each condition of two weeks' growth. Four biological replicates of this experiment were performed with qualitatively similar results.

Nitrate is the form of nitrogen source that can be used by *B. subtilis* but cannot be utilised by *S. indica*. When using nitrate as the sole nitrogen source for testing the co-culture, the interaction pattern was the same as observed in glutamine condition (Figure B-1), that is, *S. indica* colony expanded and overlapped with *B. subtilis* (Figure B-2). Notably, although *S. indica* cannot utilise nitrate, with the presence of thiamine, *S. indica* formed a thin layer of hyphae in its monoculture confirmed by microscopy. This result was consistent with previous OD_{600} measurement of *S. indica* growing on 6-well plate on Figure 2-9 in section 2.4.2.

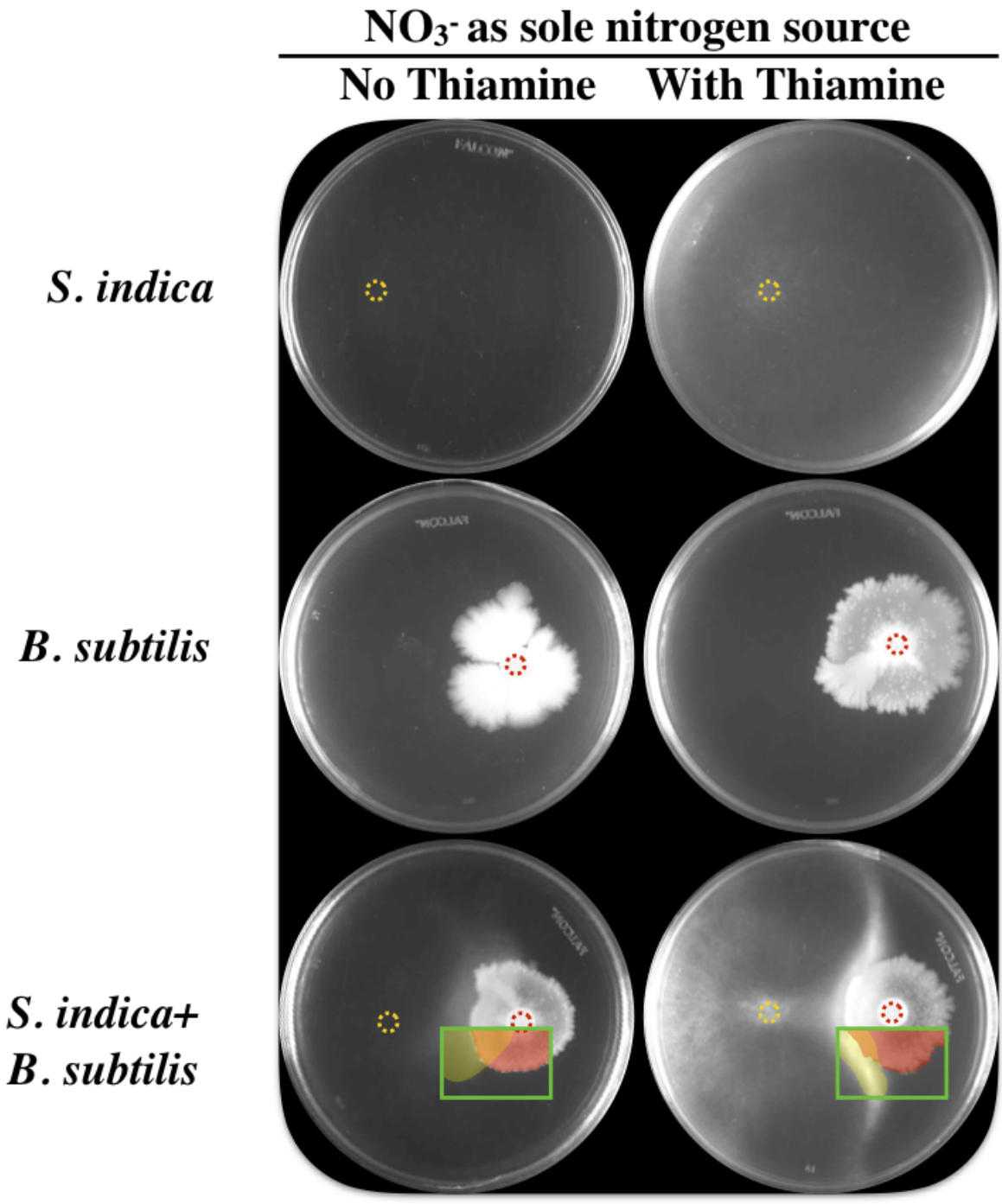


Figure B-2: *S. indica* and *B. subtilis* growth on synthetic medium containing nitrate as the sole nitrogen source. Rows from top to bottom show growth of monocultures of *S. indica*, *B. subtilis*, and their co-culture, respectively. The left and right columns show growth on medium with or without thiamine, respectively. The yellow dotted circle on the images indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis* inoculation point. The green square highlights the colonies with pseudo-colour, where the area covered by *S. indica* hyphae is shown in yellow, and the *B. subtilis* colony is shown in red. The areas were manually drawn with software keynote. When both organisms

were cultured together (bottom row), *S. indica* and *B. subtilis* were inoculated on the left and right of the plate, with a 20 mm space in between. The diameter of each plate is 60 mm. Plates shown are representative of at least three replicates for each condition of two weeks' growth. Two biological replicates of this experiment were performed with qualitatively similar results.

B.3.2 *S. indica* and *B. subtilis* interaction pattern is pH related

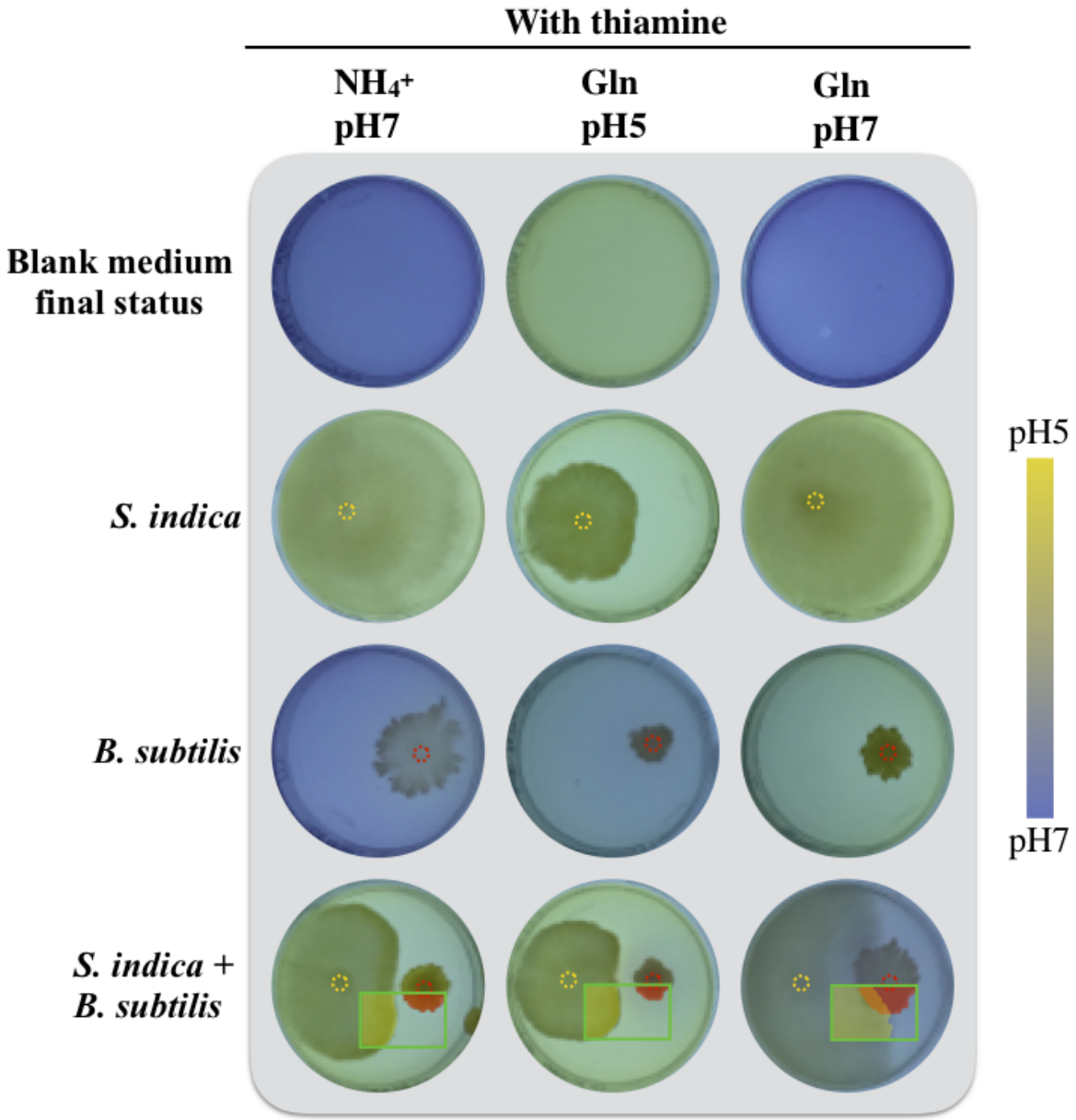
Since both organisms release organic acids when they grow, and consuming ammonium in medium leads to a pH decrease while glutamine and nitrate do not, a hypothesis was raised that the decreasing of environmental pH might influence the microbial interaction patterns. Therefore, bromocresol purple as a pH indicator was added to the synthetic medium to test this hypothesis.

The synthetic media containing thiamine and different nitrogen source (ammonium or glutamine) were used for setting up 3 different media treatment: ammonium as the sole nitrogen source with initial pH 7, glutamine as the sole nitrogen source with initial pH7, and glutamine as the sole nitrogen source with initial pH5. The same inoculation scheme was performed as mentioned in section 3.2.3. After two weeks' growth, similar interaction patterns shown in Figure 3-14 were found in treatments of initial pH 7 media (Figure 3-16), that *S. indica* avoided *B. subtilis* when ammonium was the sole nitrogen source, and *S. indica* overlapped with *B. subtilis* when glutamine was the sole nitrogen source. However, on the medium containing glutamine as the sole nitrogen source at a pH 5 initiation, the avoidance interaction pattern of co-culture was observed. On the treatments creating avoidance interaction pattern (NH_4^+ pH7 and Gln pH5 in Figure B-3), the final pH values of the media were both below 7. Where colonies overlapped (Gln pH7 in Figure B-3), the final pH of medium remained neutral. This suggested a possible correlation between the environmental pH and the interaction pattern of *S. indica* and *B. subtilis* co-culture.

The colour on blank medium of glutamine as the sole nitrogen source at pH 5 initiation was slightly above 5 after two week's incubation, possibly due to some chemical properties changing in medium after incubation.

(Figure is on next page)

Figure B-3: *S. indica* and *B. subtilis* growth on synthetic medium containing thiamine and pH indicator. Rows from top to bottom show blank medium with no inoculation (as colour control), growth of monocultures of *S. indica*, growth of monocultures of *B. subtilis*, and *S. indica* and *B. subtilis* co-culture, respectively. The left, middle and right columns show growth on media containing ammonium as the sole nitrogen source with initial pH 7, glutamine as the sole nitrogen source with initial pH 5, or glutamine as the sole nitrogen source with initial pH 7, respectively. The yellow dotted circle on the images indicates the *S. indica* inoculation point. The red dotted circle indicates *B. subtilis* inoculation point. The green square highlights the colonies with pseudo-colour, where the area covered by *S. indica* hyphae is shown in yellow, and the *B. subtilis* colony is shown in red. The areas were manually drawn with software keynote. When both organisms were cultured together (bottom row), *S. indica* and *B. subtilis* were inoculated on the left and right of the plate, with a 20 mm space in between. Colour chart on the right shows the bromocresol purple pH indication range. The diameter of each plate is 60 mm. Plates shown are representative of at least four replicates for each condition of two weeks' growth. Two biological replicates of this experiment were performed with qualitatively similar results.


With thiamine
NH4+ pH7
Gln pH5
Gln pH7
Blank medium final status
S. indica
B. subtilis
S. indica + B. subtilis
pH5
pH7

B.4 Discussion

The co-cultures of *S. indica* and *B. subtilis* have specific interaction patterns (colonies avoidance or overlapping) in accordance with the provided nitrogen sources. This observation was further linked to the changing of pH when colonies developed. The actual causes of such interaction variation could be revealed with further investigation such as untargeted metabolic analysis, extracellular enzyme activities, or gene expression levels. The interaction patterns could provide insights for setting up successful co-cultures of these microbes during potential agricultural applications such as seed pre-treatment or biofertilizer.

Appendix C

Cultivate *A. thaliana* with *S. indica* and *B. subtilis*

C.1 Introduction

In order to explore the potential plant growth promoting effect of the synthetic microbial community consists of *S. indica* and *B. subtilis*, preliminary tests were done to associate *Arabidopsis thaliana* with the synthetic community.

Arabidopsis thaliana ecotype Col-0 was used for preliminary tests. *Arabidopsis thaliana* is a small flowering plant widely used as a model organism in plant biology (Rhee *et al.*, 2003). Ecotype (geological race or accession) Col-0 (or Columbia) is a wild-type of *A. thaliana* wildly used for its high fertility and vigorousness. The whole genome of *A. thaliana* has been reported (Vessey, 2003) and mutants of various functions have been reported (Kaul *et al.*, 2000; Swarbreck *et al.*, 2008). *A. thaliana* also has thiamine biosynthesis ability (Kong *et al.*, 2008), which makes it a potential candidate to study thiamine auxotrophy interaction with *S. indica*. Trials have been made to culture *A. thaliana*, *S. indica* and *B. subtilis* together on same medium, and to test the *A. thaliana* root exudates to *S. indica* growth.

C.2 Materials and methods

Analytical-grade chemicals were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA), or Fisher Scientific UK (Loughborough, UK).

C.2.1 Surface sterilisation of *Arabidopsis thaliana* seeds

It is necessary to use surface sterilised seeds for the experiments involving *A. thaliana*, so that no other microbial contamination was brought to the system with *A. thaliana* seeds.

A. thaliana seeds were obtained from Dr Patrick Schäfer lab. Seeds were put in a sterile 2 ml tube (not to exceed one-fourth of the tube volume) to be mixed with 1ml sterile tween water (200 µl tween 20 in 1 liter MilliQ water) by briefly shaking. Seeds and tween water were mixed well by flicking the tube. Seeds were spun down briefly with a centrifuge (3000 g for 10 s). Then the tween water was pipetted out and replaced with 1 ml 70 % (v/v) ethanol. Seeds and ethanol were mixed well by flicking the tube and spun down briefly. Ethanol was then replaced with 1 ml NaOCl solution (15 ml of commercial Domestos bleach and 10 ml sterile MilliQ water, resulting in approximate 3% NaOCl). The tube was inverted and occasionally flicked during 5 mins. Seeds were then rinsed 8 times with sterile water (seeds spun down and the liquid replaced with sterile water). Washed seeds were transferred to 0.2 % (w/v) Gelrite solution for sowing on plates.

C.2.2 Growing *A. thaliana* on solid medium

A square petri dish (120 long x 120 wide x 17 height mm, Ref 688161, Greiner) was used for preparing plates, keeping *A. thaliana* upright position during growth (Figure C-1). *A. thaliana* salts medium (Wilson *et al.*, 1990) (Table C-1) without added sucrose from the original recipe (ATS) was used to create optimum growth condition for *A. thaliana*. Gelrite in ATS medium serves as a solidifying agent, for a higher clarity for light penetration than using the agar.

Table C-1: ATS medium

ATS medium	1 L
Gelrite	4.5 g
KNO_3	0.505 g / 1 M stock solution: 5 ml
KH_2PO_4	0.31 g / 1 M stock solution: 2.5 ml
K_2HPO_4	0.04 g / 1 M stock solution: 2.5 ml
$MgSO_4$	0.74 g / 1 M stock solution: 3 ml
$Ca(NO_3)_2$	0.71 g / 1 M stock solution: 3 ml
Fe-EDTA	18 mg / 20 mM stock solution: 2.5 ml
H_3BO_3	4.328 mg /
$MnCl_2$	2.77 mg /
$CuSO_4$	0.125 mg / 1000X microelement stock solution: 1 ml
$ZnSO_4$	179.5 µg /
Na_2MoO_4	48 µg /
NaCl	584 µg /
$CoCl_2$	2.38 µg /

To prepare plates for culturing *A. thaliana* (Figure C-1), Gelrite was measured and mixed with 200 ml MilliQ water containing all other medium ingredients. This mixture was stirred or shaken vigorously to disperse Gelrite. The mixture was then brought to 1 liter and autoclaved. After autoclave, the medium was poured onto square petri dishes before it solidified. Each square petri dish holds approximate 80 ml medium. After medium solidified, a sterile spatula was used to cut out a slice of 1 cm medium at one side of each plate. This was to create a headspace so that *A. thaliana* shoots and leaves had room to grow (Figure C-1). *A. thaliana* seeds were weighed to determine the approximate numbers according to needs (1000 seeds equal approximately 20 mg). Seeds of a certain amount (according to experiments) were taken to surface sterilisation procedure described in method section C.2.1. Sterilised seeds transferred with sterile cut pipette tip (cut 10 µl pipette tip so that its top has 2 mm opening allowing seeds to enter) were sowed on plates, below the cut line of the

headspace and on the surface of the medium (Figure C-1). The headspace of each plate was kept at the top when stacking the plates. The plates with *A. thaliana* seeds were tied together with stationary tape and were covered with cling film only at the top, to avoid potential contamination while allowing aeration (Figure C-1). The plates were incubated at 4 ℃ for 2 days in a cold-room for seeds stratification.

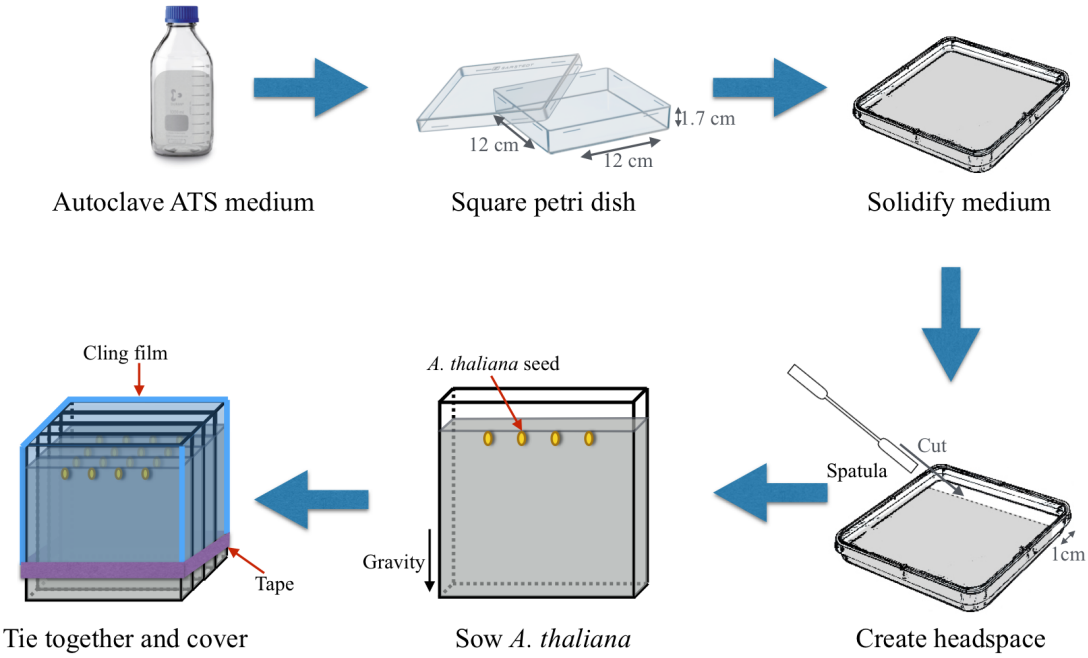


Figure C-1: Illustration of ATS plate preparation and *A. thaliana* seeds sowing procedure. The detailed procedure is described in C.2.2. ATS medium is prepared and autoclaved. Liquid ATS medium is poured into square petri dishes for solidifying. The solidified plates were cut with a spatula for creating head space. *A. thaliana* seeds are sowed on these plates. Plates are tied together with cling film covering their tops.

C.2.3 Growing *Arabidopsis thaliana* on synthetic medium

A. thaliana growth was initially tested on the developed thiamine-free synthetic medium containing ammonium or nitrate as the sole nitrogen source and compared with growth on ATS medium (positive control) and ATS medium containing 20 g/l glucose. This test followed cultivation method described in section

C.2.2, with sowing 15 seeds evenly distributed at a horizontal line below the headspace on each plate. Plates were incubated in the environmental chamber (Sanyo versatile environmental test chamber) at 10-hour photoperiod and a temperature cycle of 22 °C/18 °C (day/night). After two weeks, no seed germinated on these medium containing glucose, while the seeds on ATS medium all germinated.

Due to no germination of seeds on medium containing glucose, another test was done with the glucose-free medium. Thiamine-free synthetic media containing ammonium, glutamine or nitrate as sole nitrogen were used for testing *A. thaliana* growth, while ATS medium served as positive control. This test followed cultivation method described in section C.2.2, with sowing 24 seeds evenly distributed at a horizontal line below the headspace on each plate. Plates were incubated in the environmental chamber (Sanyo versatile environmental test chamber) at 10-hour photoperiod and a temperature cycle of 22 °C/18 °C (day/night). After 3 weeks, images of plates were recorded with a commercial camera (8-megapixel camera with *f*/2.2 aperture). Germinated *A. thaliana* were manually counted, and the root length of each plant was determined by manually outlining and calculating plate images using Fiji (ImageJ).

C.2.4 Growing *S. indica*, *B. subtilis* and *A. thaliana* together

ATS medium and ATS medium with additional 150 μg/l thiamine were used to perform this experiment. *A. thaliana* seeds were sowed at 1 spots on each plate with 8 seed per spot following the cultivation method described in C.2.2. Plates with seeds were incubated in the environmental chamber (Sanyo versatile environmental test chamber) at 10-hour photoperiod and a temperature cycle of 22 °C/18 °C (day/night) for 10 days.

S. indica (spore suspension prepared as described in section 2.3.2) was inoculated 1 µl on every plate, 1 cm to the right of *A. thaliana* root, 3 cm below headspace. One microliter of *B. subtilis* (culture prepared as described in section 3.3.2), or 10 mM $MgCl_2$ (as mock treatment), or 100 mg/L thiamine was inoculated 2 cm below the *S. indica* inoculation point respectively (Figure C-2). *A. thaliana* with *S. indica* and mock in ATS medium serves as control. All plates were put back to environmental chamber after inoculation and continued incubation condition for 15 days.

Images of plates were recorded with a commercial camera (8-megapixel camera with *f*/2.2 aperture). Magnified images were taken by combining commercial camera (8-megapixel camera with *f*/2.2 aperture) with Nikon eclipse e200 microscope and Nikon MRP20102 10× objective.

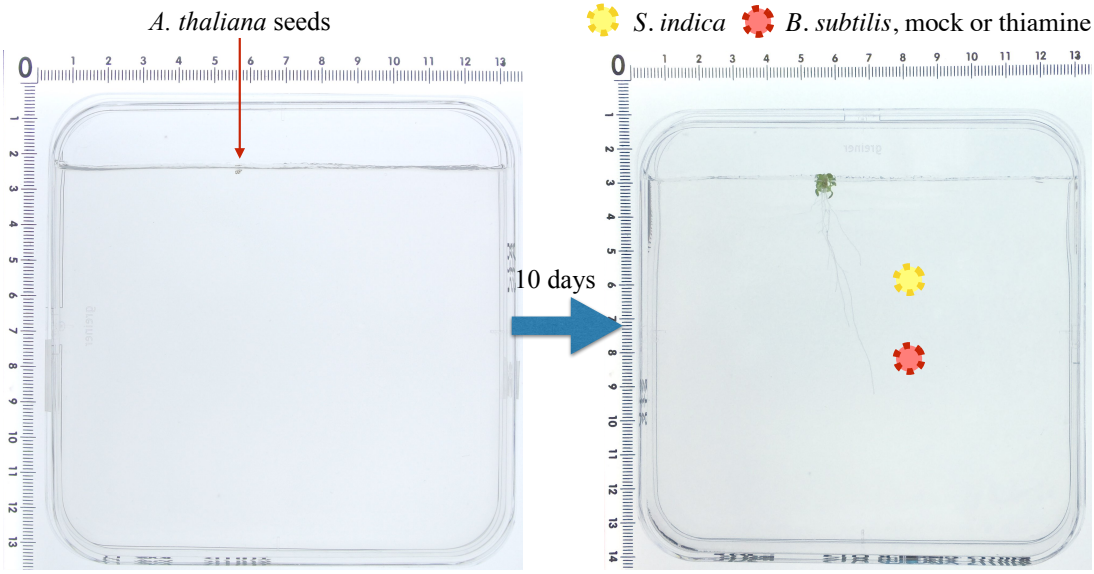


Figure C-2: Inoculation scheme of *S. indica*, *B. subtilis* and *A. thaliana* on plate. The detailed procedure is described in section C.2.4. Left plate indicates where *A. thaliana* seeds are sowed. Right plate indicates where *S. indica* and *B. subtilis* are inoculated.

C.2.5 Growing *S. indica* on *A. thaliana* root exudates

ATS medium was used for growing *A. thaliana*. *A. thaliana* seeds were sowed 10 seeds evenly distributed at a horizontal line below the headspace on each plate, following the cultivation method described in C.2.2. Plates were incubated in the environmental chamber (Sanyo versatile environmental test chamber) at 10-hour photoperiod and a temperature cycle of 22 °C/18 °C (day/night) for 20, 30 and 40 days. At 20, 30 or 40 days after incubation, one plate was taken out for preparing the root exudates. Root exudates were retrieved by peeling off the *A. thaliana* from ATS plates, cutting the rest of the Gelrite medium into 1 cm^2 pieces, and putting pieces into 50 ml sterile centrifugation tubes (conical polypropylene centrifuge tube, Ref 352070, Falcon) (Figure C-3). The tube containing Gelrite pieces were centrifuged at 13000 g for 20 min. Afterwards, the pieces were pressed, and most root exudates were in the supernatant. The supernatant was collected for filter sterilisation and then frozen at -20 °C. Once all 3 time-points root exudates were collected, previously frozen samples were thawed at room temperature.

Medium for testing *S. indica* was prepared by mixing an equal volume of synthetic medium (ammonium as the sole nitrogen source, containing 3 % of agar to make 1.5 % final agar concentration) and root exudates from *A. thaliana* growing for 20 days, 30 days and 40 days respectively. The same synthetic medium was also mixed with equal volume of ATS medium (as negative control), with an equal volume of water (as negative control), and with an equal volume of ATS medium containing 150 μg/l thiamine (as positive control), respectively. Three-microliters of each test medium was put on each well of the 6-well plates (Ref: 353046, Falcon). One microliter of *S. indica* spore suspension (prepared as described in section 2.3.2) was inoculated at the centre of each well. Plates were incubated at 30°C in a static

incubator for 2 weeks. Images were taken using gel doc system (U: Genius 3, Syngene).

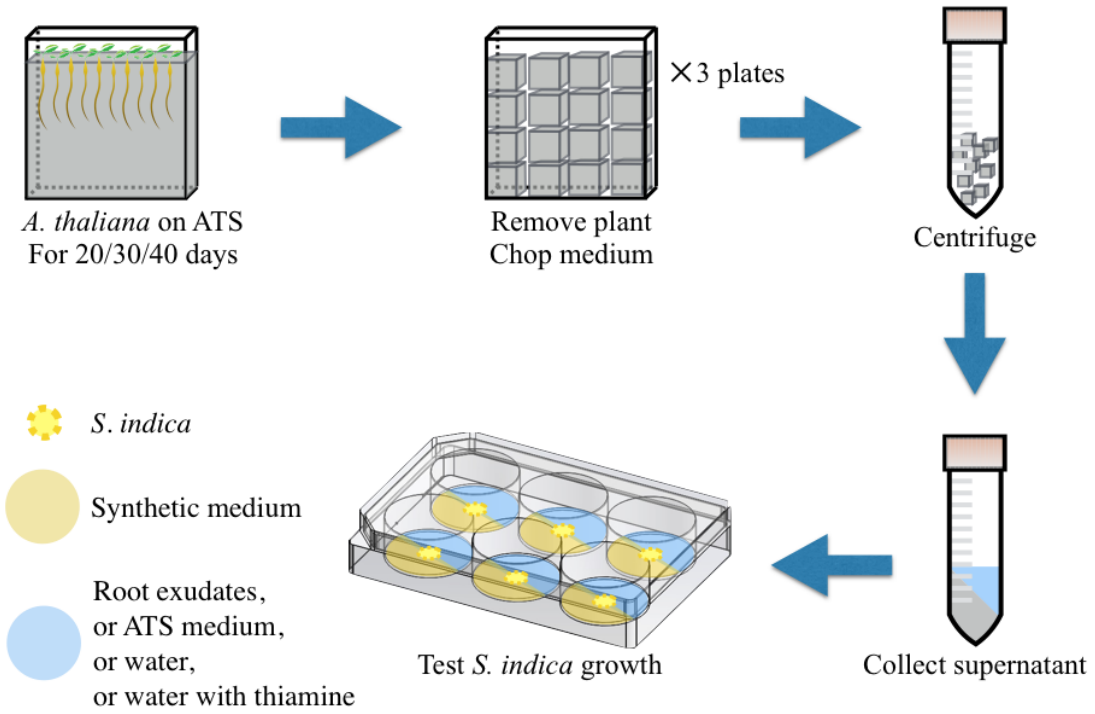


Figure C-3: Schematic of *A. thaliana* root exudates preparation for *S. indica* growth test. The detailed procedure is described in section C.2.5. *A. thaliana* plates of different ages are chopped into pieces. These pieces are collected and centrifuged to obtain the supernatant. The supernatant is mixed with different media for testing *S. indica* growth.

C.2.6 Growing *S. indica*, *B. subtilis* and *A. thaliana* together on medium containing different nitrogen source

A. thaliana seeds were sowed 20 seeds evenly distributed at a horizontal line below the headspace on 6 ATS plates, following the cultivation method described in C.2.2. Plates with seeds were incubated in the environmental chamber (Sanyo versatile environmental test chamber) at 16-hour photoperiod and a temperature cycle of 22 °C/18 °C (day/night) for 7 days.

Germinated *A. thaliana* of same growth status (similar length of roots with white surface and no wilt) were chosen and transferred to a new plate containing ATS

medium and ATS ammonium medium. On each plate, three *A. thaliana* were placed at the centre of each plate with 5 mm space in between (Figure C-4). These plates were then incubated in the same environmental chamber for one day.

S. indica (spore suspension prepared as described in section 2.3.2) and *B. subtilis* (culture prepared as described in section 3.3.2) were used for inoculating on the plates containing *A. thaliana*. Two schemes of *S. indica* inoculation were performed: "spread" and "drop". In the "spread" scheme, 100 µl of *S. indica* spore suspension was applied along the roots; in the "drop" scheme, 10 µl of *S. indica* spore suspension was inoculated 4 cm below the centre of roots (Figure C-4). Two schemes of *B. subtilis* inoculation were also performed: at the same time with *S. indica*, or 3 days after *S. indica* inoculation. Both cases *B. subtilis* were inoculated as a 10 µl drop placed at 4 cm below the centre of roots (Figure C-4). For the control, 10 µl of 10mM $MgCl_2$ was applied 4 cm below *A. thaliana* roots.

These inoculated plates were sealed individually with the surgical tape (Micropore, Ref 1530-25, 3M), and incubated in the same environmental chamber for 2 more weeks (starting from *S. indica* inoculation). At the end of incubation, plates were scanned using a commercial scanner (HP). Fresh shoot weight was measured by cutting the shoots from the stem of each plant, and the weight was measured using a precision balance (Sartorius).

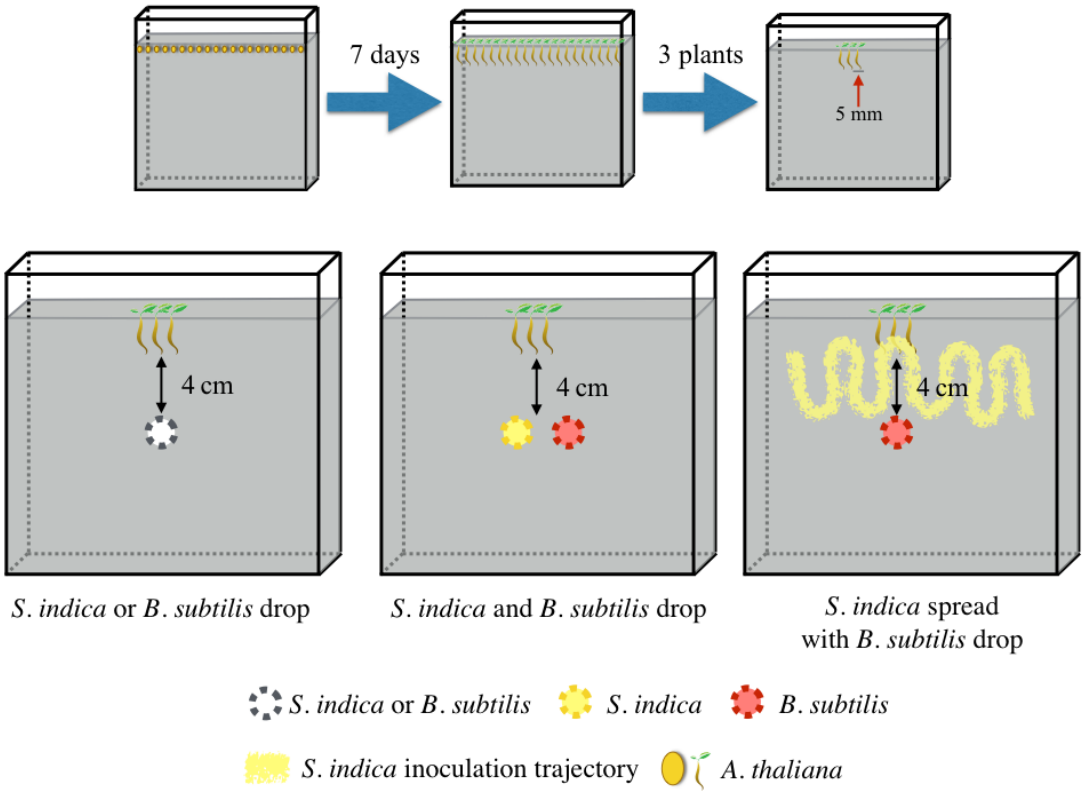


Figure C-4: Inoculation scheme of *S. indica*, *B. subtilis* and *A. thaliana* on plates. The detailed procedure is described in section 3.3.14. *A. thaliana* seeds were germinated on ATS medium. Healthy *A. thaliana* plants of same length were selected and put onto fresh ATS plates. *S. indica* and *B. subtilis* are inoculated on these plates following different inoculation schemes.

C.3 Results

C.3.1 Synthetic medium cannot support *A. thaliana* growth

When *A. thaliana* seeds sowed on synthetic media containing ammonium or nitrate as the sole nitrogen source, no germination was observed after two weeks. As a contrast, the same batch of seeds all germinated on ATS medium. This could due to the glucose from synthetic medium delayed *A. thaliana* seed germination, as reported in other studies (Dekkers *et al.*, 2004; Zhu *et al.*, 2009).

Therefore, *A. thaliana* was grown again on glucose-free synthetic media, containing ammonium, glutamine or nitrate as the sole nitrogen source. Germination rates and root lengths were recorded. Although on all tested media sowed seeds showed similar good germination (Figure C-5 A), the root length representing plant growth conditions was very different (Figure C-5 B). *A. thaliana* growing on the glucose-free synthetic medium containing nitrate as the sole nitrogen source produced 2.7 times shorter roots than growing on the positive control ATS medium. Among glucose-free synthetic media containing different nitrogen source, nitrate generated around 2.8 times and 2.3 times longer roots than ammonium and glutamine respectively; root lengths from ammonium and glutamine media were not significantly different. Therefore, the synthetic medium or the glucose-free synthetic medium cannot support good growth of *A. thaliana*.

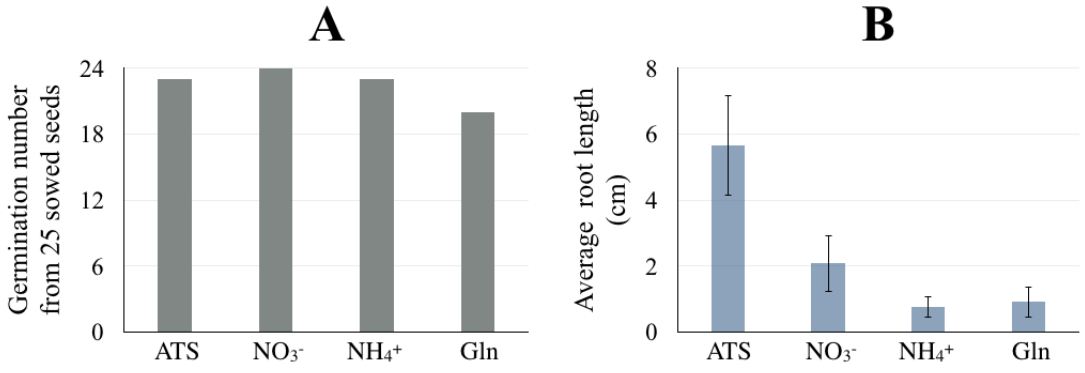


Figure C-5: *A. thaliana* root length and number of germinated seeds on different media. ATS indicates ATS medium (the positive control); NO_3^- indicates glucose-free synthetic medium containing nitrate as the sole nitrogen source; NH_4^+ indicates glucose-free synthetic medium containing ammonium as the sole nitrogen source; Gln indicates glucose-free synthetic medium containing glutamine as the sole nitrogen source. **(A).** Germination numbers of 25 sowed *A. thaliana* seeds on each medium treatment. **(B).** *A. thaliana* root length after two weeks' growth on different media. The root lengths from 24 seeds under each treatment are used for generating the length data and standard deviation error bars. This experiment is done only once due to the time limitation of this project.

C.3.2 The preliminary test on *S. indica*, *B. subtilis* and *A. thaliana* tri-culture

Both *B. subtilis* and *A. thaliana* produce thiamine (Helliwell *et al.*, 2013). Therefore, the thiamine auxotrophy of *S. indica* could contribute in shaping the interaction among the three organisms. Preliminary tests were done to cultivate *S. indica*, *B. subtilis* and *A. thaliana* together, to understand whether *S. indica* growth has any preference towards different thiamine source.

Due to the fact that synthetic medium cannot support good growth of *A. thaliana* (section C.3.1), ATS medium was used for creating a shared environment for the three organisms. ATS medium with additional 150 µl/l thiamine was used to create an environment without thiamine limitation as a comparison. *S. indica* and *B. subtilis* (or thiamine droplet) were inoculated by the side of *A. thaliana* root. However, *S. indica* did not form eye-recognisable colony comparing to previous growth test on synthetic medium (Figure 2-7), neither did it show any growth tendency towards *B. subtilis*, *A. thaliana* (Figure C-6) or thiamine droplet. Under the microscope, *S. indica* was found to form a thin layer of hyphae across the whole plate in all treatments, while *B. subtilis* covered *A. thaliana* root hairs with biofilm (Figure C-7).

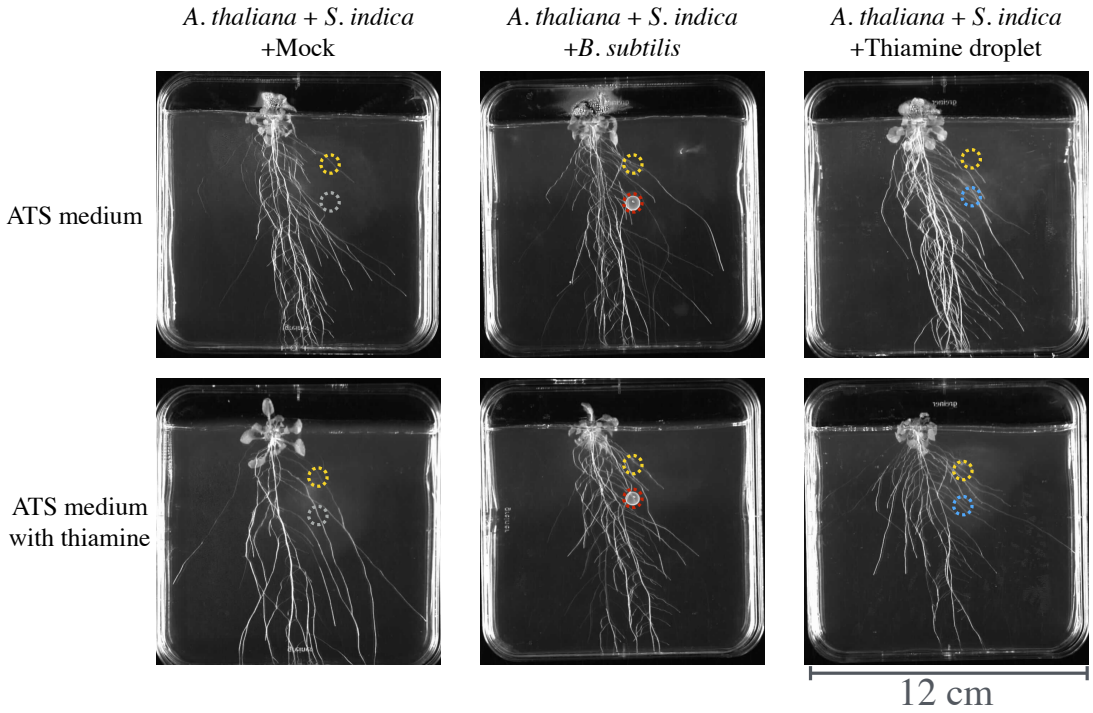


Figure C-6: Tri-culture of *S. indica*, *B. subtilis* and *A. thaliana* on the ATS medium and ATS medium containing thiamine. Grey dotted circle indicates mock treatment (10mM $MgCl_2$) inoculation point; yellow dotted circle indicates *S. indica* inoculation point; red dotted circle indicates *B. subtilis* inoculation point; blue dotted circle indicates thiamine droplet inoculation point. *A. thaliana* appears as white leaves in headspace and while roots across plates. *A. thaliana* with *S. indica* and mock in ATS medium serves as control. Plates shown are representative of seven replicates for each treatment of 25 days old *A. thaliana* and 15 days old *S. indica* and *B. subtilis* on the same plate.

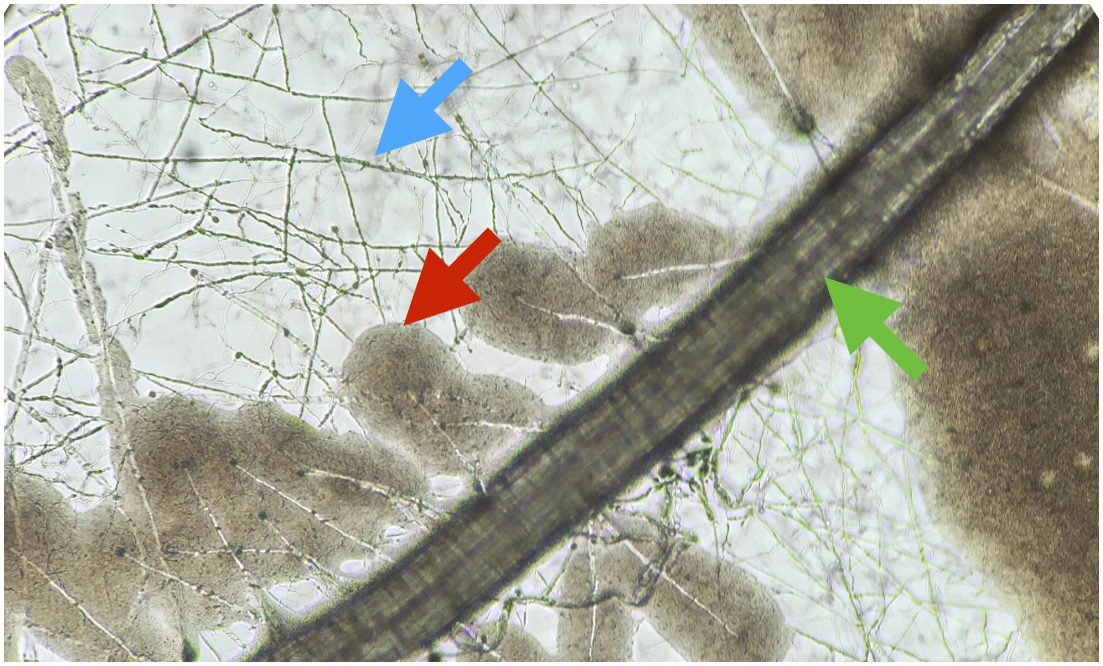


Figure C-7: Microscopic view of *S. indica*, *B. subtilis* and *A. thaliana* on ATS medium. Blue arrow points to hyphae of *S. indica;* red arrow points to *B. subtilis* biofilm covering *A. thaliana* root hair; green arrow points to *A. thaliana* secondary root. Image shown is the details from the plate presented in Figure C-6 (treatment: *S. indica*, *B. subtilis* and *A. thaliana* on ATS medium).

C.3.3 The preliminary test on *S. indica* growing on *A. thaliana* root exudates

Evidence was found that thiamine level in root exudates decreased during *S. indica* colonising *A. thaliana* root (Strehmel *et al.*, 2016). To understand whether *S. indica* could get thiamine from the plant through root exudates rather than having to be associated with plants, the preliminary test was done in cultivating *S. indica* on *A. thaliana* root exudates.

To eliminate the effect of lacking other nutrients such as a carbon source, *S. indica* was cultivated on a mixture of synthetic medium (thiamine-free, ammonium as the sole nitrogen source) together with root exudates. Root exudates from different ages of *A. thaliana* (20-day, 30-day and 40-day old), together with ATS medium, water or ATS medium containing thiamine as controls were used for mixing with synthetic medium to create different treatments. However, *S. indica* only showed

growth on the positive control, the synthetic medium mixing with ATS medium and thiamine (Figure C-8). No eye-recognizable *S. indica* growth were observed on any medium treatment containing root exudates (Figure C-8).

However, this experiment cannot conclude whether *S. indica* gets thiamine from colonising *A. thaliana* or from *A. thaliana* root exudates. The non-growth of *S. indica* due to low thiamine concentration might be caused by root exudates containing a limited amount of thiamine, or the technique used for exudate collection (described in method section C.2.6) causing thiamine degradation.

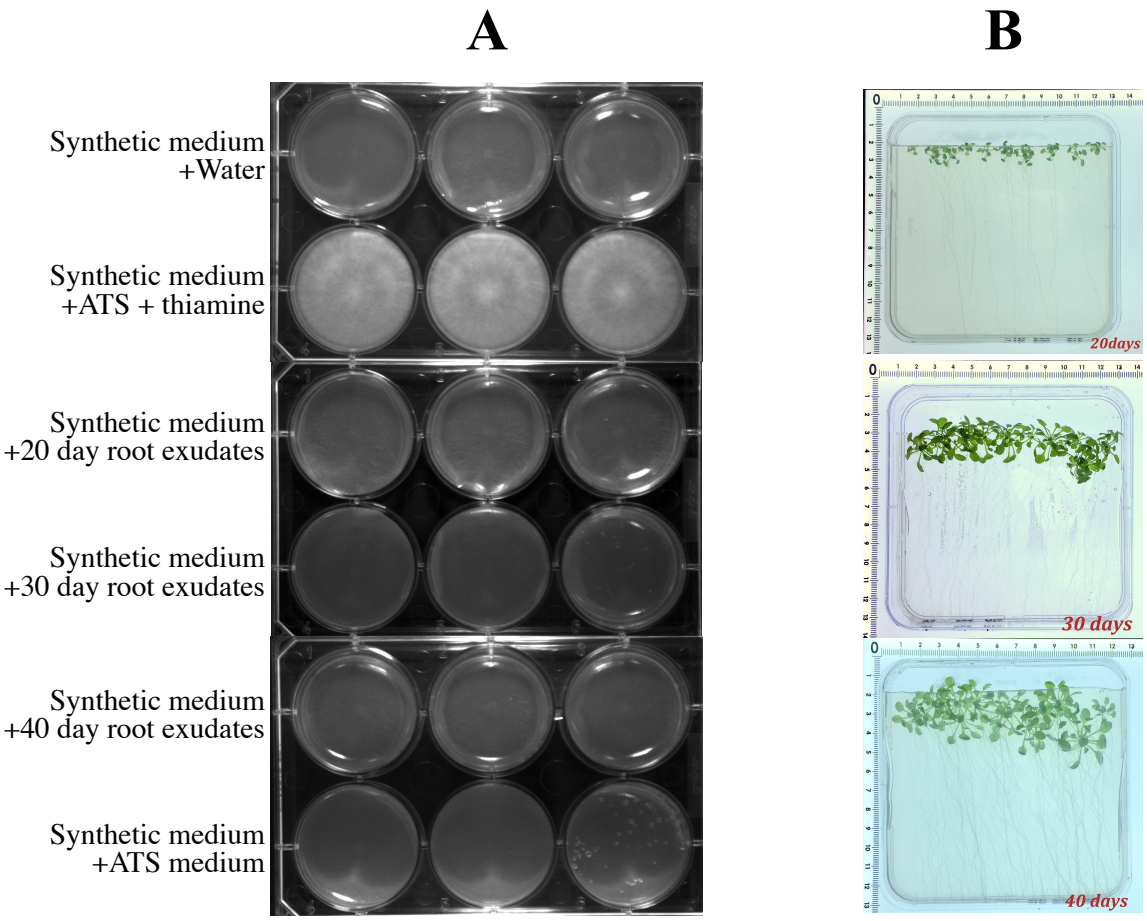


Figure C-8: *S. indica* growth test on *A. thaliana* root exudates. **(A).** Two weeks' growth of *S. indica* growing on different media treatments. "Synthetic medium + Water" indicates the medium containing equal volumes of synthetic medium (thiamine-free, ammonium as the sole nitrogen source) and water, as the negative control. "Synthetic medium + ATS + thiamine" indicates the medium containing equal volumes of synthetic medium and ATS medium containing 150 µg/l thiamine (final thiamine

concentration in medium is 75 μg/l), as the positive control. "Synthetic medium + 20/30/40 day root exudates" indicates the medium containing equal volumes of synthetic medium and root exudates from 20/30/40-day old *A. thaliana* plates, respectively; each well contains the root exudates from an individual *A. thaliana* plate represented in **(B)**. "Synthetic medium + ATS medium" indicates the medium containing equal volumes of synthetic medium and ATS medium, as the negative control. *S. indica* grows in white colonies. Each treatment has three replicates presented in three adjacent wells. **(B).** *A. thaliana* growth on ATS medium for different time spells. From top to bottom are the 20-day, 30-day and 40-day old *A. thaliana*. Plates shown are representative of three replicates of the same age *A. thaliana*.

C.3.4 The preliminary test on *S. indica-B. subtilis* community potential growth promoting effect on *A. thaliana*

Studies have shown ammonium inhibits *A. thaliana* growth (Britto and Kronzucker, 2002; Qin *et al.*, 2011). Therefore, ammonium was used to replace the nitrate in the ATS medium as the nitrogen source to both create a possible stress condition for *A. thaliana*, and to investigate the potential plant growth promoting effect from the *S. indica-B. subtilis* community.

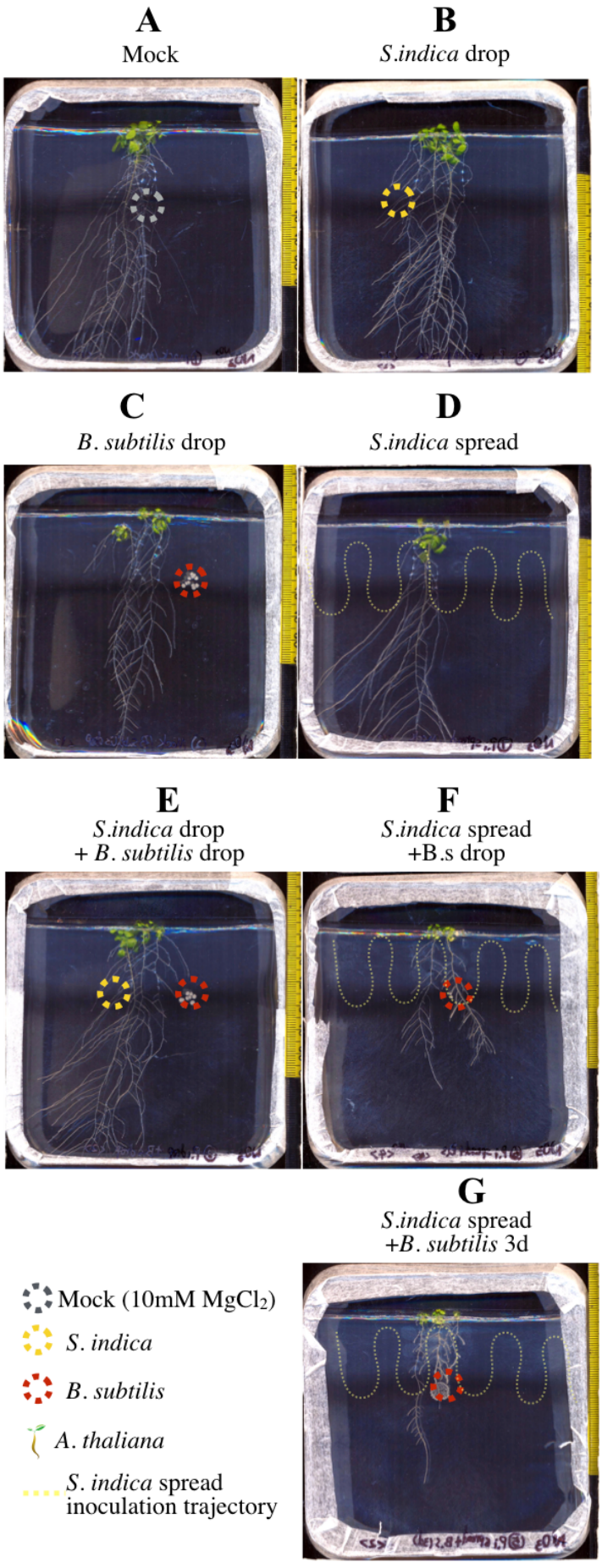
Seven-day-old healthy *A. thaliana* plants of similar root length originally cultivated on ATS medium was transferred onto fresh ATS medium and ATS ammonium medium for testing growth with *S. indica* and *B. subtilis*. *S. indica* and *B. subtilis* were applied to *A. thaliana* in different ways. 1) *S. indica* was inoculated as a droplet 4 cm below the *A. thaliana* roots (Figure C-9 B, E); or applied along roots for better coverage on root surface (Figure C-9 D, F, G). *B. subtilis* were inoculated as a droplet 4 cm below the *A. thaliana* roots (Figure C-9 C) and 2 cm horizontal to *S. indica* droplet (Figure C-9 E); as a droplet 4 cm below the *A. thaliana* roots while *S. indica* was applied along roots (Figure C-9 F); as a droplet 4 cm below the *A. thaliana* roots 3 days after *S. indica* was applied along roots (Figure C-9 G) to allow higher

chance of colony establishment due to microbes overlapping. Four technical replicate plates were used for each treatment. Due to the fact that *A. thaliana* roots intertwined and were difficult to measure, shoot weights were recorded two weeks after inoculation of *S. indica*.

However, among three biological replicates on this experiment (Figure C-10), no consistent results were obtained to indicate *A. thaliana* was stressed from the ATS ammonium medium; nor did the results demonstrate any growth promoting effect (such as the increase of fresh shoot weight) to *A. thaliana* from *S. indica* or *B. subtilis*.

(Figure is on next page)

Figure C-9: *A. thaliana* on ATS medium and ATS ammonium medium under different microbial treatments. Grey dotted circle indicates mock treatment (10mM $MgCl_2$) inoculation point; yellow dotted circle indicates *S. indica* inoculation point; red dotted circle indicates *B. subtilis* inoculation point; yellow dotted line indicates *S. indica* inoculation trajectory. *A. thaliana* appears as green leaves in headspace and while roots across plates. Each plate contains 3 *A. thaliana* of similar initial status placed at the centre with 5 mm space in between. **(A).** Mock (control) treatment (10mM $MgCl_2$) 10 µl applied 4 cm below *A. thaliana* roots when initiating plates. **(B).** *S. indica* spore suspension 10 µl applied 4 cm below *A. thaliana* roots when initiating plates. **(C).** *B. subtilis* culture 10 µl applied 4 cm below *A. thaliana* roots when initiating plates. **(D).** *S. indica* spore suspension 100 µl applied along *A. thaliana* roots when initiating plates. **(E).** *S. indica* spore suspension 10 µl and *B. subtilis* culture 10 µl applied 4 cm below *A. thaliana* roots, 2 cm in between when initiating plates. **(F).** *S. indica* spore suspension 100 µl applied along *A. thaliana* roots, while *B. subtilis* culture 10 µl applied 4 cm below *A. thaliana* roots when initiating plates. **(G).** *S. indica* spore suspension 100 µl applied along *A. thaliana* roots when initiating plates, and *B. subtilis* culture 10 µl applied 4 cm below *A. thaliana* roots 3 days after *S. indica* inoculation. Plates shown are representative of four replicates on ATS ammonium medium for each treatment of 3 weeks old *A. thaliana* and 2 weeks old *S. indica*. Three biological replicates were performed but no consistent results obtained (Figure 3-26). Therefore, this image is only for representing microbial treatments layout.


A
Mock
B
S.indica drop
C
B. subtilis drop
D
S.indica spread
E
S.indica drop
+ B. subtilis drop
F
S.indica spread
+B.s drop
G
S.indica spread
+B. subtilis 3d
Mock (10mM MgCl2)
S. indica
B. subtilis
A. thaliana
S. indica spread
inoculation trajectory

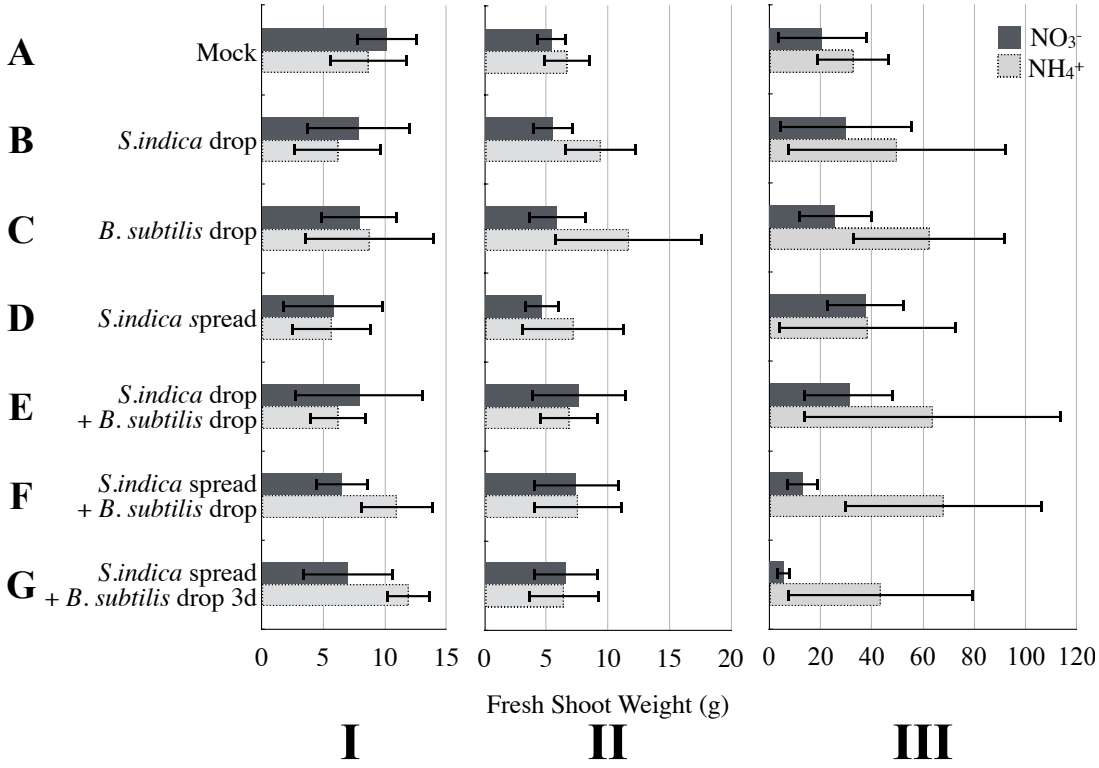


Figure C-10: Fresh shoot weights of *A. thaliana* on ATS medium and ATS ammonium medium under different microbial treatments. **(A)-(G)** correspond to the microbial treatments showing in Figure 3-25. Four technical replications were used for generating the weight data and standard deviation error bars. **(I)**, **(II)** and **(III)** are data collected from three different biological replicates, with no consistent results.

C.4 Discussion

Both *S. indica* and *B. subtilis* live in soil, and many of their activities are involved with plants (Pandey and Palni, 1997; Varma *et al.*, 1999). *S. indica* and *B. subtilis* are associated to *A. thaliana* as model systems respectively to study plant-microbe interactions (Peškan-Berghöfer and Shahollari, 2004) (Vlamakis *et al.*, 2013). Therefore, the *S. indica-B. subtilis* community was expected to perform plant beneficial effects in this study. However, establishing *A. thaliana* in the synthetic medium was challenging, and associating *S. indica-B. subtilis* system to *A. thaliana* using the *A. thaliana* medium (ATS) did not generate expected results.

These preliminary tests involving *A. thaliana* were challenging to design, as the three organisms have different growth paces (*A. thaliana* in weeks, *S. indica* in days and *B. subtilis* in hours to develop a mature colony), and nutritional preference (such as pH, nitrogen or carbon source and so on). The conventional way of inoculating microbes to *A. thaliana* root on solid medium plates did not provide consistent effects to show their physiological interactions. A possible explanation on the lack of plant growth promoting effect showing in section C.3.4 could be that ATS is an optimum growth medium for *A. thaliana*, small adjustment on the recipe would not produce significant differences. Consequently, *A. thaliana* grew well enough on ATS medium and ATS ammonium medium without experiencing any stress. The potential plant growth promoting effect would not appear while *A. thaliana* was already in optimal growth. Alternatively, the applied microbe quorum could be relatively low and insufficient to induce the desired positive effect.

Unlike many microorganisms, plants growth is gravitropism, meaning that plant roots are growing toward the centre of the earth or gravitational pull (positive gravitropism) and the shoot growing away from it (negative gravitropism) (Migliaccio

and Piconese, 2001). Cultivation of *A. thaliana* requires concern on gravity comparing to the cultivation of *S. indica* or *B. subtilis*. Recording these plant plates was therefore, difficult to go down to the resolution of detailed microbe distribution with available microscopy device in this study.

Both *B. subtilis* and *A. thaliana* produce thiamine (Helliwell *et al.*, 2013). To understand if *S. indica* has any preference on getting thiamine from *B. subtilis* or *A. thaliana*, experiment was done on culturing *S. indica*, *B. subtilis* and *A. thaliana* together and record *S. indica* growth direction. However, this experiment did not consider other nutritional limiting factors affecting *S. indica* or *B. subtilis* growth, such as insufficient carbon source from 10 days old *A. thaliana* when inoculating microbes. The unmeasurable results did not provide information on whether *S. indica* has a preference on the thiamine source. Since all three organisms established on the same plate, the tests showing in section C.3.2 suggested a potential way of cultivating *S. indica*, *B. subtilis* and *A. thaliana* together.